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Metabolomic and chemometric profiles of *Tribulus terrestris* L. from three different locations in Mpumalanga province, South Africa

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Background: *Tribulus terrestris* L. is a traditional herb regularly recognised as puncture vine, yellow vine, devils horn, goat head and caltrop, this is often a yearly shaggy herbaceous plant species with stems of up to 2m long, having a place to the family of Zygophyllaceae.

Aim: The study explored the therapeutic potential of this herb as it is being utilised for pharmaceutical purposes because of its furostanol saponins, which have a stimulating impact on characteristic testosterone levels.

Setting: The study took place in different locations of Mpumalanga Province, Bushbuckridge (24.8398°S, 31.0464°E), Kamagugu (25.4566° S, 31.0034° E) and Nkomatipoort (25.4510° S, 31.9587° E).

Methods: Utilising ultra-high-performance liquid chromatography quadrupole time-of-flight mass spectrometry (UPLC-QTOF/MS), 50 metabolites were tentatively identified within the leaves of Tribulus terrestris L. from three diverse areas of Mpumalanga Province, South Africa.

Results: Metabolomic-chemometric analysis revealed that Parvispinoside B, F-Gitonin and Gitonin scored highest for the discrimination of *Tribulus terrestris* L. from three locale clusters. Heat maps showed designs and groupings based on the metabolite concentrations.

Conclusion: This study provided novel insights in terms of thorough identification of the secondary metabolites and characterization of the leaves of *Tribulus terrestris* L. in the areas studied.

Contribution: To the best of our knowledge, this study presents the first metabolite profile of *Tribulus terrestris* L. and its compositional differences in the Mpumalanga region, providing chemical-based evidence for its nutritive and/or health benefits

Keywords: gitonin; steroidal saponins; chemometric; UPLC-QTOF/MS; medicinal plants; PCA; heat map.

Introduction

Tribulus terrestris L. is a traditional herb regularly recognised as puncture vine, yellow vine, devil's horn, goat head and caltrop; this is often a yearly shaggy herbaceous plant species with stems of up to 2 m long. It belongs to the family of Zygophyllaceae (Hashim et al. 2014; Zhu et al. 2017). *T. terrestris* L. is an indigenous plant species that thrive in warm, humid climates in Africa, southern Europe, Australia and western and southern Asia (Hashim et al. 2014). In addition, it is broadly spread throughout Africa, Japan, Korea, some parts of Asia and China (Sharifi, Darabi & Akbarloo 2003). *T. terrestris* L. grows best in light-textured soils and is found in abandoned areas and along the roadside, cultivated crops, gardens, and overgrazed pasture (Hashim et al. 2014). The plant is well-known for its yellow petal bloom and prickly natural product, while the leaves are pinnate, short and inverted, with 4–8 sets of spear-shaped leaflets on each leaflet. The fruits have a rough surface, are axe-shaped and are normally 6 mm long, with lengths ranging from 7 mm to 12 mm (Zhu et al. 2017).

The seeds are encased in carpels (a woody star-shaped structure) around 5 mm – 7 mm and 5 mm – 6 mm in length and width, respectively. Each carpel comprises approximately five seeds, and each seed is yellow and almost 3 mm long. The root is stringy, slim, round, hollow,

frequently branched, light brown and bearing numerous small rootlets (Chhatre et al. 2014). *T. terrestris* L. is used as a palliative, tonic, stomachic, antihypertensive, sexual enhancer, urinary disinfectant, astringent, diuretic and lithotriptic in traditional medicine (Chhatre et al. 2014). The fruit of *T. terrestris* L. has been employed in Chinese pharmacopoeia as a cough expectorant that improves eyesight, as a diuretic and for tonifying the kidney, as a cure for skin itch, vertigo and headache, and as a treatment for mammary duct obstruction (Zhu et al. 2017). In India, however, it is used to treat infertility, impotence, low libido, and erectile dysfunction.

Numerous pharmacological studies have revealed *T. terrestris* L. to have antimicrobial activity, antiurolithic activity, diuretic activity, aphrodisiac activity, antidiabetic activity, immunomodulatory activity, antioxidant activity absorption enhancement and central nervous system activity (Chhatre et al. 2014; Hashim et al. 2014; Kianbakht & Jahaniani 2003). Moreover, it was detailed that the antimicrobial activity of *T. terrestris* L. varies with the plant part utilised and its origin. For instance, the spirostanol saponins of Iranian and Indian *T. terrestris* L. were reported to exhibit antifungal activity against *Cryptococcus neoformans* and *Candida albicans*. In addition, the leaves, fruit and roots of Iraqi *T. terrestris* L. were stated to have antimicrobial activity against 11 species of pathogenic and nonpathogenic micro-organisms' rootlets (Chhatre et al. 2014). The dominant saponins of *T. terrestris* L. are thought to be spirostanol and furostanol. *T. terrestris* L.'s steroidal saponins are thought to be the plant's active ingredients, and the amount and variety of these compounds mostly depend on the environment (Dinchev et al. 2008). The saponins of *T. terrestris* L. from different geographic areas vary in terms of composition and content (Kostova & Dinchev 2005). To date, 58 and 50 kinds of spirostane and furostane saponins have been recognised from *T. terrestris* L., respectively, while flavonoids in *T. terrestris* L. are the derivatives of quercetin, isorhamnetin and kaempferol (Yang et al. 2014). For example, kaempferol-3-*O*-rutinoside, quercetin-3-*O*-gent-7-*O*-glu and isorhamnetin-3-*O*-gent-7-*O*-glu are flavonoids with kaempferol, quercetin and isorhamnetin as the basic parent structure, respectively, while tribulusimide C, harmine, tribulusterine, tribulusin A, etc. are the main alkaloids isolated from the leaves, fruit and stems of *T. terrestris* L. (Ren et al. 1994). Among the distinctive sort of compounds, flavonoids and steroidal saponins are observed as the foremost crucial metabolites with different bioactivities. Chemometric analysis is widely used in metabolomics research to characterise and assess the diversity of bioactive metabolites found in useful foods. Furthermore, a phytochemical database for bioactive compounds in foods that can be linked to chemical properties associated with nutritional and nutraceutical effects is required (Farag et al. 2019). As a result, the purpose of this study was to look into the various metabolites in *T. terrestris* L. from different locations in Mpumalanga province using ultra-high-performance liquid chromatography (UHPLC) coupled with a high-resolution quadrupole time-of-flight mass spectrometry (Q-TOF-MS) technique and a chemometric analysis approach.

Research methods and design

Plant material

Fresh leaf samples of *T. terrestris* L. were separately harvested from three different locations of Mpumalanga province, Bushbuckridge (24°50′23.28′′S, 31°2′47.04′′E), Kamagugu (25°27′23.76′′S, 31°0′12.24′′E) and Nkomatipoort[1] (25°27′3.6′′S, 31°57′31.32′′E). The samples were harvested between October and December 2020. All the harvested samples were growing in the locations as weeds (not cultivated). To minimise variation, young tender leaves, specifically from the apex of the shoot, free of dirt or soil particles, decay and/or damage, were carefully plucked from 20 young flowering plants according to the method reported by Ntuli (2019), with modifications. To prevent contamination and moisture loss, the samples were put in Ziploc™ bags. On arrival in the laboratory, the samples were separately washed under running distilled water and freeze-dried (−85°C, LyoQuest-55/Telstar, Shanghai, China) and ground into fine powder for analysis according to the method reported by Moloto et al. (2020).

Extraction of leaf extracts for ultra-high-performance liquid chromatography quadrupole time-of-flight mass spectrometry analysis

Freeze-dried leaf samples (0.1 g) were homogenised in water with 2 mL of 80% methanol, then sonicated at 30°C for 30 min. The mixture was then centrifuged at 3000 × g for 20 min at 4°C using a Hermle Z326k (Hermle Labortechnik GmbH, Wehingen, Germany). The collected supernatants were used for the UHPLC-QTOF-MS analysis.

The ultra-high-performance liquid chromatography quadrupole time-of-flight mass spectrometry profiling and quantification

A 0.22-µm polytetrafluorethylene filter was then used to filter the supernatants. A Quadrupole 120 time-of-flight (QTOF) mass spectrometer UPLC-QTOF/MS (Waters, Milford, Massachusetts, United States of America [USA]) was used to identify and quantify predominant secondary metabolites. An ACQUITY UPLC BEH C18 column (2.1 mm × 100 mm i.d., 1.7 × 10^{-6} m; Waters) was used for all analyses. The mobile phase was composed of acetonitrile (A) and 0.1% formic acid, v/v (B), with the following gradient elution: (1) 0–8 min, 95% – 80% A; (2) 8–12 min, 80% – 70% A; (3) 12–15 min, 70 A – 65 A; (4) 15–18 min, 65% A; (5) 18–21 min, 65% – 20% A; (6) 21–23 min, 20% – 5% A; (7) 23–24 min, 5% A; (8) 25–30 min, 95% A. The flow rate of the mobile phase was 0.4 mL/min and the temperatures of the column and autosampler were

1.Nkomati, is short for Nkomatipoort and will be used throughout the article.

maintained at 30°C and 10°C, respectively. Data were processed using Mass Spectrometry – Data Independent Analysis (MS-DIAL) and MS-FINDER (RIKEN Center for Sustainable Resource Science: Metabolome Informatics Research Team, Kanagawa, Japan) (Lai et al. 2018; Tsugawa et al. 2015). Functions 1 (unfragmented channel) and 2 (fragmented channel) of the Waters MSe data were processed by MS-DIAL to produce MS1 and MS2 spectra as well as extracted ion chromatograms with associated peak height intensity data. As calibration standards are not available for the majority of these compounds, the peak height intensity was converted to concentration in a semiquantitative manner by interpolation of a calibration curve for catechin acquired under the same instrumental conditions. Each deconvoluted feature (alignment in MS-DIAL), together with its associated MS1 and MS spectra, were exported from MS-DIAL to MS-FINDER. Based on the accurate mass elemental compositions, possible compounds were identified from the listed databases and then subjected to *in silico* fragmentation. According to the spectral match between the *in silico* and measured spectra, a score (out of 10) is assigned to each of the possible compound matches, with the highest score being accepted as the most likely (assuming a score of at least four).

Chemometric data analysis

The UPLC-QTOF-MS data of *T. terrestris* L. from different locations of Mpumalanga province were analysed with MetaboAnalyst 5.0 (Xia Lab @ McGill, Ste-Anne-de-Bellevue, Quebec, Canada) using principal component analysis (PCA), partial least squares – discriminant analysis (PLS-DA) and variable importance in projection (VIP) scores and a heat map. To evaluate the PLS-DA model's goodness-of-fit, Q^2 (predictive ability) and R^2 (cumulative interpretation ability) were taken as initial indicators. Using a VIP plot, significantly different metabolites were screened.

Ethical considerations

This article followed all ethical standards for research without direct contact with human or animal subjects.

Results and discussion

Identification and characterisation of secondary metabolites using ultra-high-performance liquid chromatography quadrupole time-of-flight mass spectrometry

Secondary metabolites that could be the elements driving the antiproliferative effect of *T. terrestris* L. leaves were identified by nontargeted profiling using ultra-performance liquid chromatography coupled with high-resolution Q-TOF-MS. The UHPLC mass spectrometry (MS) examination of *T. terrestris* L. leaves taken from several locations in Mpumalanga province revealed 50 secondary metabolites, the majority of which were flavonoids, phenolic acids and saponins (Table 1). The annotations or identifications were tentative based on accurate mass elemental composition and fragmentation spectra. Based on ESI positive data, the results from this study revealed that quercetin derivatives (quercetin 3-gentiobioside and rutin) and F-gitonin with concentrations of 10886, 9307 and 7296.7 mg/kg DW, peak 7; 9068, 7118 and 5885.3 mg/kg DW, peak 9; and 10387, 7613.5 and 5707.7 mg/kg DW, peak 23 in Kamagugu, Nkomati and Bushbuckridge, respectively, were predominant compounds in the leaves of *T. terrestris* L. Peak 39, m/z 426.301, was discovered as compound jervine in the leaves of *T. terrestris* L., which had the lowest concentrations (296.7, 440, and 422.5 mg/kg DW) from Bushbuckridge, Kamagugu and Nkomati, respectively. The leaves of T. terrestris L., as reported in this study, were high in steroidal saponins, which are compounds likely to be responsible for the libido effects in humans and possibly the testosterone mimicry in animals (Ștefănescu et al. 2020). Enrichment of the saponins in *T. terrestris* L. reported in this study poses them as an ideal carbohydrate source for diabetic patients because of their ability to potentiate glucose-induced insulin release (Pereira-Leal & Seabra 2001). Saponins, because of their amphiphilic molecule, have membrane-permeabilising properties; thus, they could increase the absorption of other compounds. This property is of great importance because toxic effects could appear in patients with multiple conditions who undergo chronic treatments. The current findings contrast with those of Lazarova et al. (2011), who found significant differences (different amounts of chemicals and the absence of some compounds) between samples taken from the same geographic location. A rise in the use of *T. terrestris* L. supplements has also been seen among athletes, who are always looking for natural ways to improve their performance (Ștefănescu et al. 2020). Saponins have biological and therapeutic activities that include: (1) haemolytic factor, (2) anti-inflammatory, (3) antibacterial, (4) antifungal, (5) antiviral, (6) insecticidal, (7) anticancer, (8) cytotoxic and (9) molluscicidal effects (Abdel Gawad 1999; Armelle et al. 2018; Cheng et al. 2011; Ellen et al. 2007; Hassan et al. 2010; Just et al. 1998; Simões, Amoros & Girre 1999; Sindambiwe et al. 1998; Sparg, Light & Van Staden 2004).

As a result of the complexity and multidimensionality of metabolomic data, appropriate statistical and chemometric tools were used to obtain chemical information and convert it into biological knowledge (Boccard & Rudaz 2014). Chemometrics is the science of extracting valuable information from complex data sets through pattern recognition and machine learning algorithms (Brereton 2015; Tebani, Afonso & Bekri 2018). The unsupervised (PCA) obtained data from the UPLC-QTOF/MS showed that the phenolic metabolites in the leaves of *T. terrestris* L. from different locations of Mpumalanga province differed in distribution. Principal component analysis is a multivariate technique that increases interpretability and minimises the loss of biological information by reducing the dimensionality

TABLE 1: Tentative identification of secondary metabolites in *Tribulus terrestris* from three different locations of Mpumalanga province using ultra-high-performance liquid chromatography quadrupole time-of-flight mass spectrometry data.

Peak	Retention time (min)	Tentative identification	Molecular formula	MS/MS fragments	Class	[M-H]-	Bushbuckridge	Kamagugu	Nkomati
1	2.325	Sesbanimide A	$C_{15}H_{21}NO_7$	–	Piperidinediones	310.1285	1860.7	2940.5	2484.5
2	2.785	Quercetin 3,7-diglycoside	$C_{33}H_{40}O_{22}$	462, 301, 299	Flavonoid-3-O-glycosides	789.2102	751.7	1145	725.5
3	2.928	(R)-Salsolinol	$C_{10}H_{13}NO_2$	–	Tetrahydroisoquinolines	180.1024	285	543	386.5
4	3.04	Brassicoside	$C_{34}H_{42}O_{22}$	–	Flavonoid-7-O-glycosides	803.2237	507.7	1072	668.5
5	3.312	Tuberonic acid	$C_{12}H_{18}O_4$	207.07309, 207.02063	Jasmonic acids	227.1285	3921.7	5781	5234.5
6	3.327	Quercetin 3,7-diglycoside+xyl/ quercetin 3-sambubioside-7-glucoside	$C_{32}H_{38}O_{21}$	759.20184, 759.17285	Flavonoid-7-O-glycosides	759.199	643.7	1174.5	919
7	3.515	Quercetin 3-gentiobioside	$C_{27}H_{30}O_{17}$	151.0649, 179.04198, 301.02756, 303.49591, 463.10941, 463.75592	Flavonoid-3-O-glycosides	627.1572	7296.7	10 886	9307
8	3.656	Elemicin	$C_{12}H_{16}O_3$	93.066778, 110.07061, 135.08803, 165.0847, 168.98448, 193.98524	Anisoles	209.1183	2375.3	3368.5	2883.5
9	3.76	Rutin	$C_{27}H_{30}O_{16}$	151.98985, 271.0224, 301.20242	Flavonoid-3-O-glycosides	611.161	5885.3	9068	7118
10	3.812	Indolelactic acid	$C_{11}H_{11}NO_3$	–	Indolyl carboxylic acids and derivatives	20.0807	1119.7	1566.5	1436
11	3.828	Isorhamnetin 3-glucosyl-(1->6)-galactoside	$C_{28}H_{32}O_{17}$	–	Flavonoid-3-O-glycosides	641.1718	1661	2689.5	2160.5
12	3.907	Beta-damascenone	$C_{13}H_{18}O$	–	Enones	191.1437	458.3	733.5	629.5
13	3.967	10 beta-12,13-dinor-8-oxo-6-eremophilen-11-al	$C_{13}H_{18}O_2$	–	Cyclohexenones	207.1385	453.3	703	603.5
14	4.028	Indolylacryloylglycine	$C_{13}H_{12}N_2O_3$	–	N-acyl-alpha amino acids	245.0931	1081.7	1719.5	1424.5
15	4.286	Loliolide	$C_{11}H_{16}O_3$	–	Benzofurans	197.1174	3871	5255.5	4767.5
16	4.359	Parvispinoside A	$C_{56}H_{94}0_{29}$	–	Steroidal saponins	1213.588	2763	3699	35395
17	4.849	Parvispinoside B	$C_{56}H_{94}0_{28}$	593.47076, 595.39264, 757.35364, 1079.60864	Steroidal saponins	1197.5858	1592.7	3486.5	2496
18	6.315	Dihydroactinidiolide	$C_{11}H_{16}O_2$	79.0531, 79.31911, 91.04725, 107.05179	Benzofurans	181.1222	3905.7	5345	45215
19	6.474	Nonivamide	$C_{17}H_{27}NO_3$	–	Methoxyphenols	294.2076	1499.3	1560.5	1513.5
20	6.591	Funtumine	$C_{21}H_{35}NO$	–	Pregnane steroids	318.2786	900.3	896	925
21	6.965	[8]-Shogaol	$C_{17}H_{24}O_3$	–	Shogaols	277.181	637	952	717.5
22	7.295	Terrestrosin D	$C_{50}H_{80}O_{23}$	–	1,3-aminoalcohols	1071.4984	1208.7	1734.5	1367
23	7.806	F-gitonin	$C_{50}H_{82}O_{23}$	289.08154, 433.25568, 755.35333, 757.48743, 1051.62415	Steroidal saponins	1051.5314	5707.7	10 387.5	7613.5
24	8.166	Terminaline	$C_{23}H_{41}NO_2$	–	Gluco- and mineralocorticoids, progestogens and derivatives	364.3211	4975.3	7010.5	6195.5
25	8.299	Ebelactone B	$C_{21}H_{36}O_4$	–	Terpene lactones	353.2697	1327.7	1943.5	1661.5
26	8.469	3-Oxo-2-(2-entenyl) cyclopentaneoctanoic acid	$C_{18}H_{30}O_3$	–	Prostaglandins and related compounds	317.2085	1792.7	2646	2239.5
27	8.653	Gitonin	$C_{50}H_{82}O_{22}$	–	Steroidal glycosides	1035.54	1000.7	1832	1268.5
28	8.736	Methyl(octadecyl)amine	$C_{19}H_{41}N$	–	–	284.3323	386.3	972.5	471.5
29	8.932	6-(14-methoxytetradecyl)-2-methylpiperidin-3-ol	$C_{21}H_{43}NO_2$	–	Alkaloids and derivatives	342.337	2525.3	3386.5	2749
30	8.991	Desglucouzarin	$C_{29}H_{44}O_9$	–	Cardenolide glycosides and derivatives	537.3044	440	861.5	568.5
31	9	N-undecylundecan-1-amine	$C_{22}H_{47}N$	–	Dialkylamines	326.3779	1077.3	1621.5	1145.5
32	9.4	Terpendole F	$C_{28}H_{39}NO_4$	–	Naphthopyrans	454.2923	968.3	1658	1247
33	9.511	Alpha-linolenoyl ethanolamide	$C_{20}H_{35}NO_2$	–	N-acylethanolamines	322.2735	969	1227	1064
34	10.213	5-O-methylembelin	$C_{18}H_{28}O_4$	–	P-benzoquinones	309.2046	1001.3	979.5	1016
35	10.578	N-dodecyl-N-methyldodecan-1-amine	$C_{25}H_{53}N$	–	Trialkylamines	368.4254	975	936	958
36	10.638	Linoleamide	$C_{18}H_{33}NO$	–	Fatty amides	280.263	1763	2418.5	1897
37	10.703	Sphingosine	$C_{18}H37NO_2$	–	1,2-aminoalcohols	300.2905	485	659	562.5
38	10.718	Tetrahydrobungeanool	$C_{18}H_{33}NO_2$	–	N-acyl amines	296.2578	466.3	582	462
39	10.718	Jervine	$C_{27}H_{39}NO_3$	–	Jerveratrum-type alkaloids	426.301	296.7	440	422.5
40	10.721	Gamma-linolenic acid	$C_{18}H_{30}O_2$	–	Linoleic acids and derivatives	279.2329	1975.7	2409	2145
41	10.724	Trihexyphenidyl	$C_{20}H_{31}NO$	–	Aralkylamines	302.2449	933.7	1659.5	1098.5
42	10.738	2-[(2-decoxyphenyl) carbamoyl]benzoic acid	$C_{24}H_{31}NO_4$	–	Tetrahydroisoquinolines	398.2329	3208	3197.5	3242.5
43	10.751	Linoleamide	$C_{18}H_{33}NO$	–	Fatty amides	280.2628	1397.7	1812.5	1385
44	10.854	Camptothecin	$C_{20}H_{16}N_2O_4$	–	Camptothecins	349.1204	1677.7	1725	1643

Table 1 continues on the next page →

TABLE 1 (Continues...): Tentative identification of secondary metabolites in *Tribulus terrestris* from three different locations of Mpumalanga province using ultra-high-performance liquid chromatography quadrupole time-of-flight mass spectrometry data.

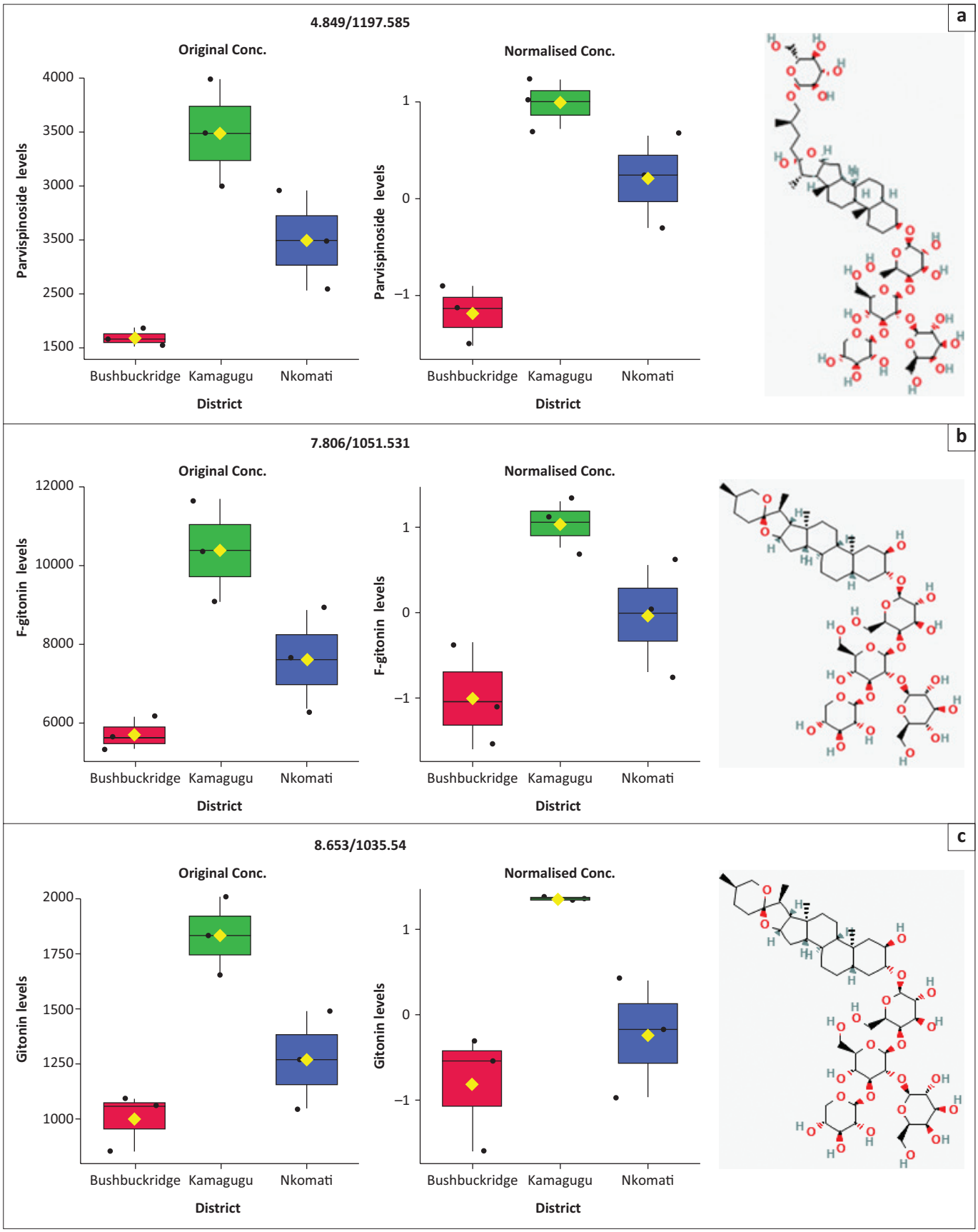
Peak	Retention time (min)	Tentative identification	Molecular formula	MS/MS fragments	Class	[M-H]-	Bushbuckridge	Kamagugu	Nkomati
45	10.86	Spiranthol A	$C_{20}H_{22}O_3$	–	Hydrophenanthrenes	333.1474	865	880	969
46	11.029	Octocrylene	$C_{24}H_{27}NO_2$	–	Diphenylmethanes	362.2113	4054	3794.5	3816.5
47	11.256	Oleamide	$C_{18}H_{35}NO$	–	Fatty amides	282.2794	23254.7	33242	26150
48	11.357	Gypsogenic acid	$C_{30}H_{46}O_5$	–	Triterpenoids	487.3427	367.7	470.5	480.5
49	11.869	Notoamide J	$C_{21}H_{25}N_3O_4$	–	Alpha amino acids and derivatives	384.193	1327	1605.5	1512
50	12.061	Octadecanamide	$C_{18}H_{37}NO$	283.2875	Carboximidic acids	284.2945	3420	4277	3597

MS/MS, tandem mass spectrometry.

of complex data sets (Jolliffe & Cadima 2016). The underlying structures and characteristics of the data are thus revealed by this unsupervised, explorative method. The PCA plot from this study revealed three district clusters based on the metabolites shown by the leaves of *T. terrestris* L. in different locations of Mpumalanga province (Bushbuckridge, Kamagugu and Nkomati village) (Figure 1 and Figure 2). In this study, the sample size was too small to develop robust chemometric models. However, as the aim was not to develop a prediction model but merely to investigate inter- and intraspecies variation, chemometric analysis was nevertheless used to analyse the liquid chromatography mass spectrometry (LC-MS) data. The two principal component (PC) models were able to distinguish clearly between the locations, with 22.9% separation along PC2 and 37.6% separation along PC1. Two locations, namely Bushbuckridge and Kamagugu, were distinctly separated by the first principal component (PC1), with Bushbuckridge samples situated on the negative (PC1) and Kamagugu on the positive (PC1) areas of the plot, while one sample from the Nkomati locality clustered along the negative (PC1), and the rest clustered in the positive (PC1). The relationship of the observed variation to locality was further evaluated by performing classification and discriminant analysis using orthogonal projections to latent structures discriminant analysis (OPLS-DA). This supervised pattern recognition technique was used to identify variables (in this case, peaks on the chromatogram) responsible for the differences between the groups. To obtain more in-depth information regarding the differential metabolic profiles revealed by PCA modelling, a supervised method, OPLS-DA, was used. It can be seen from the plot that the greatest variation was along PC2 (Figure 3). The OPLS-DA scores plot confirmed that the samples from different locations were very different in composition. Figure 4 shows the box-and-whisker plot representing an in-depth, semiquantitative visualisation of the distribution of selected metabolites ([A] parvispinoside B, [B] F-gitonin and [C] gitonin) in the leaves of *T. terrestris* L. from three different locations of Mpumalanga province plots. The interlocation variations in metabolite profiles are illustrated by the increased or relative decrease of the mentioned metabolites. Figure 4 reveals a relative increase in the metabolite concentration of *T. terrestris* L. leaves from the Bushbuckridge region, while the leaves from the Kamagugu region revealed a decreased concentration of these metabolites. The location-based quantitative and qualitative metabolite distribution was further evidenced in the group-wise metabolite selection, biplot-based discrimination analysis and the variable importance in projection (VIP), showing metabolic markers contributing to metabolic phenotypes in Figure 4. These are secondary metabolites, which were responsible for the differentiation in PCA detected. The performance of the developed model was validated by the computed parameters 'R^2 (0.9856)' and 'Q^2 (0.9577)', indicating its covered variance and good prediction power, respectively (Figure 5). Metabolites facilitating the discrimination of the leaves of *T. terrestris* L. from three different locations are shown in Figure 4. The PLS-DA also finds the characteristics (metabolites) with the highest VIP scores. As a result, the contribution of each metabolite to group separation was calculated using VIP ratings. Variable importance in projection scores is calculated by adding the squares of the partial least squares (PLS) loadings, which measure the amount of Y-variance explained across all dimensions and the weighted sum of the PLS-regression coefficients (Carvalho et al. 2021). Thus, metabolites were ranked according to their VIP scores, and only the top metabolites with the highest VIP scores were deemed to provide the most meaningful interpretation of the data (Carvalho et al. 2021). Hierarchical cluster analysis was performed on the whole data set of discovered metabolites to visualise clusters of samples with comparative chemical composition, which gave additional evidence of the related metabolites associated with *T. terrestris* L. from different regions. The results of this study were merged with a heat map structure created from metabolite concentrations across all samples (Figure 6). Blue in the heat map denotes metabolite downregulation or lower expression, whereas red depicts metabolite upregulation or higher expression. Heat maps also make patterns that might not be evident or visible.

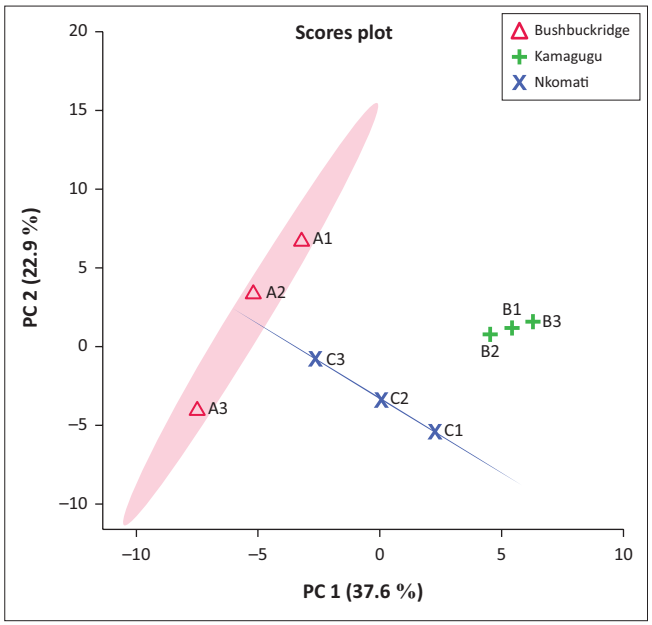
Conclusion

The results of this study indicate that the leaves of *T. terrestris* are a rich source of secondary metabolites, justifying its use in folk medicine to cure a range of diseases in various countries. The significant number of steroidal saponins in this plant is a distinctive feature. *T. terrestris* from different geographical regions had diverse secondary metabolite compositions and concentrations. According to the findings of this study, the leaves of *T. terrestris* contain therapeutic compounds and could be used to treat ailments. However, more research is needed to fully understand the



Note: These secondary metabolites were identified by ultra-high-performance liquid chromatography mass spectrometry and are responsible for the differentiation in principal component analysis. (line = mean; box = standard error; whisker = standard deviation).

FIGURE 1: Box plot showing (a) parvispinoside B, (b) F-gitonin and (c) gitonin detected in the leaves of *Tribulus terrestris* from three different locations of Mpumalanga province.



PC, principal component.

FIGURE 2: Principal component analysis score plot of principal component1 versus principal component2 scores of *Tribulus terrestris* from three different locations in Mpumalanga province, analysed via ultra-high-performance liquid chromatography quadrupole time-of-flight mass spectrometry.

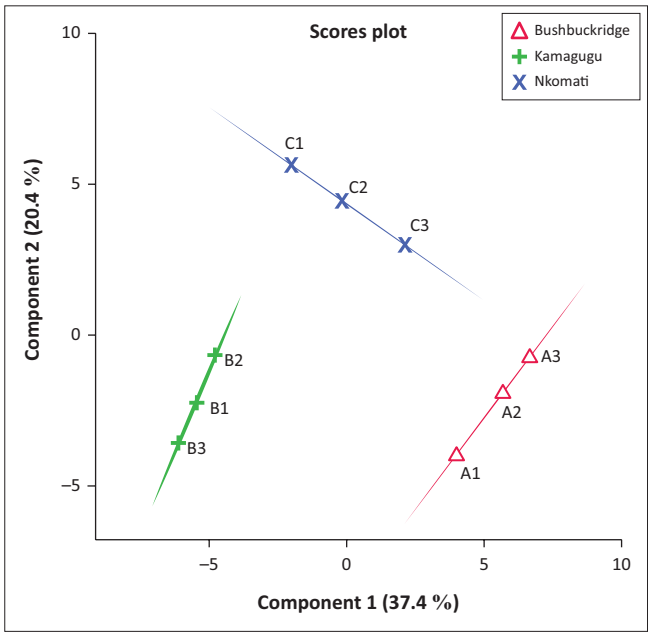


FIGURE 3: The score plot of orthogonal partial least squares discriminant analysis (OPLS-DA) of *Tribulus terrestris* from three different locations in Mpumalanga province.

pharmacological properties and biological activities associated with the described metabolite molecules.

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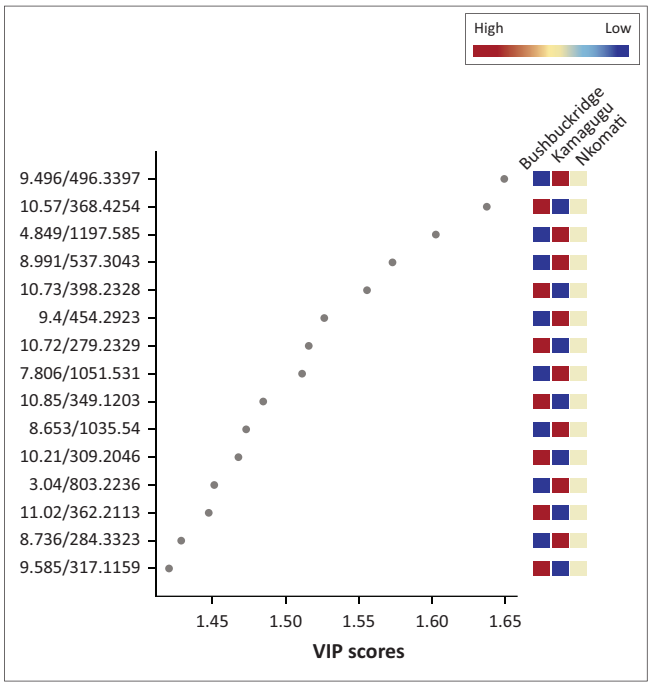
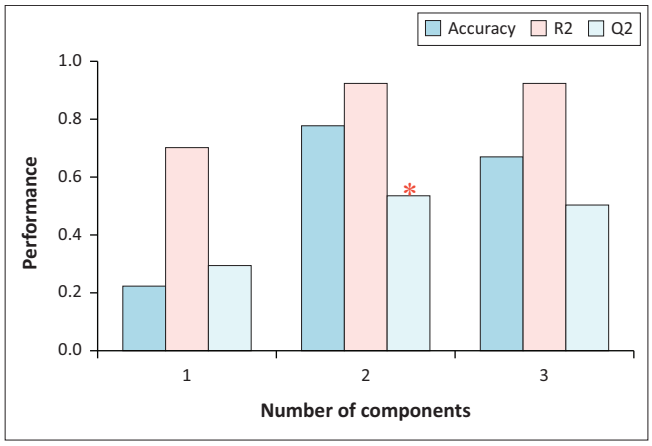


VIP, variable importance in projection.

FIGURE 4: The identification results of chemical markers of *Tribulus terrestris* from three different locations of Mpumalanga province (VIP > 3).



*, cross validation to evaluate the PLS-DA model's goodness-of-fit.

FIGURE 5: Cross validation to evaluate the PLS-DA model's goodness-of-fit, Q^2 (predictive ability) and R^2 (cumulative interpretation ability) were taken as initial indicators *T. terrestris* from three different locations in Mpumalanga province.

Competing interests

The authors declare that they have no financial or personal relationships that may have inappropriately influenced them in writing this article.

Authors' contributions

The authors designed the study, coordinated data collection, carried out all the field work and drafted the manuscript.

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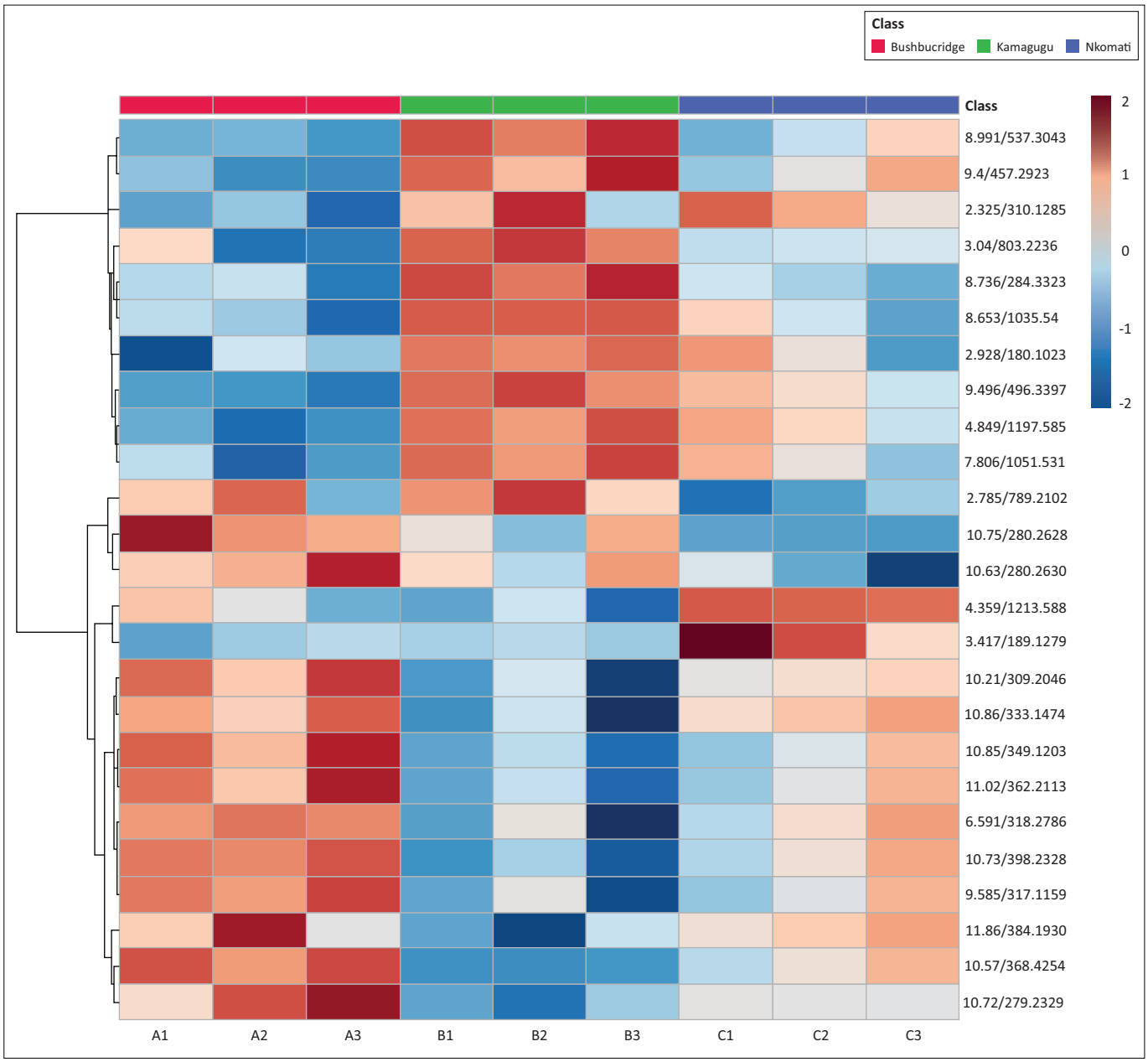

FIGURE 6: Heat map of 25 phenolic metabolites quantified in leaves of *Tribulus terrestris* from three different locations of Mpumalanga province organised in hierarchical clustering.

Data availability

Data sharing is not applicable to this article as no new data were created or analysed in this study.

Disclaimer

The views and opinions expressed in this article are those of the authors and do not necessarily reflect the official policy or position of any affiliated agency of the authors.

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