Regular Article

ANALYTICAL
AND BIOANALYTICAL
CHEMISTRY
RESEARCH

Published by the
Iranian Chemical Society

***Anal. Bioanal. Chem. Res.**, Vol. 11, No. 2, 191-199, April 2024.*

HPTLC Profiling, Quality Control and FTIR Coupled with Chemometrics Analysis for *Securidaca longipenduculata* Fresen

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(Received 14 November 2023, Accepted 24 January 2024)

In several parts of Africa, *Securidaca longipenduculata* Fresen is a medicinal plant that has become well-known in traditional medicine for its wide range of therapeutic uses. The first goal of this study was to establish a high-performance thin-layer chromatography (HPTLC) chemical fingerprint for *S. longipenduculata* roots as a reference for quality control. Methanol was used to extract the root samples that were collected from seven distinct locations in the Vhembe area of the Limpopo Province, South Africa. To resolve the major compounds from the sample matrix, an HPTLC method was optimized. The developing solvent was chloroform: ethyl acetate: methanol: formic acid (90:5:30:1), while the derivatizing agent used to view the plates was *p*-anisaldehyde/sulphuric acid. The developed HPTLC method was able to resolve the chemical constituents from the root crude material to enable clear identification of six chemical markers. The second goal was to investigate Fourier transform infrared (FT-IR) functional group information of the species in conjunction with multivariate statistical analysis. A web-based high throughput metabolomic program called MetaboAnalyst was used to build several chemometric models including principal component analysis (PCA), orthogonal projection to latent structures-discriminant analysis (OPLS-DA), and hierarchical cluster analysis (HCA). HCA analysis of data identified two clusters and additional analysis verified the quantitative differences between the samples. The FT-IR spectra of *S. longipedunculata* roots revealed a total of 15 metabolites that could be used as potential markers to differentiate between samples taken from the seven different locations.

Keywords: *Securidaca longipenduculata*, Violet tree, Quality control, HPTLC, *p*-anisaldehyde, Marker compound

INTRODUCTION

Medicinal plants have been used for centuries to combat diseases and alleviate health conditions. This form of health care is particularly popular in developing countries because medicinal plants are available without a prescription, are more affordable compared to Western medicines, and are compatible with cultural and spiritual beliefs [1]. There has been a major resurgence of interest in traditionally used medicinal plants, with a number of international and local initiatives actively exploring the botanical resources of Southern Africa, intending to identify pharmacologically active compounds. In South Africa, a large proportion of the population still relies on indigenous medicinal plants for their primary healthcare needs, often taken in conjunction with synthetic medicines [2]. According to a study, about 200000 traditional healers are reportedly consulted every day in the country for the use of medicinal plants in the treatment of critical illnesses like diabetes, tuberculosis, and malaria [3]. However, there is not enough scientific evidence to substantiate the therapeutic benefits of these plants.

Genus *Securidaca* is one of the largest, and most widely distributed genera of the family Polygalaceae, comprising about 80 species [4]. The genus occurs throughout the tropics

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of Africa with most of the species being climbers, while a few are shrubs or small trees. *Securadaca longipedunculata* Fresen, the focus of this study**,** commonly known as the Violet tree is a spiny, semi-deciduous shrub or small tree with a much-branched, open, rather straggly looking crown; that can grow from 4 - 12 metres tall [5]. The roots and stem bark of the species are taken orally either powdered or as infusions for treating chest complaints, headaches, toothaches, inflammation, tuberculosis, *etc*. [6].

The chemical composition of different plant parts the *S. Longipenduculata* has been extensively investigated and several compounds have been isolated from them, including xanthones, flavonoids, terpenes, coumarins, and steroids reported as major compounds [7]. Methyl-2 hydroxybenzoate (methyl salicylate) was isolated as a major component from the root bark volatile material [4]. From an ethyl acetate fraction of the root, additional compounds were isolated and identified, including 1,5-dihydroxy-3,4,6,7,8-pentamethoxyxanthone, 1,7-dihydroxyxanthone, 5-O-prenyl-1-hydroxy-2,3,6,7,8-pentamethoxyxathanthone, 2-hydroxy-1,7-dimethoxyxanthone, β-sitosterol, 1,7-dihyroxy-4-methoxyxanthone, quercetin-3-O-β-galacto-pyranoside and 3-hydroxy-6-methoxysalicylic. In addition, securinine, presenegenin, and 2-hydroxybenzoate esters (methyl 2-hydroxy-6-methoxybenzoate and its benzyl analogue) were also reported. In general, most classes of compounds have been isolated from the roots, using a variety of solvents [8-10].

Violet tree is widely used and amongst the most traded traditional medicine in South Africa as well as in Zambia, Botswana, Kenya, and Senegal, yet little is known regarding the phytochemical variation within the species as well as identifying marker compounds that can be used for quality control of the plant. Composition consistency is an aspect that is crucial to the commercialization of herbal medicines and their products as well as an important factor in quality control [11,12].

Quality control (QC) of herbal products can be done using a range of chromatographic fingerprinting techniques. These methods include gas chromatography (GC) with mass spectrometry (MS), thin layer chromatography (TLC) with semi-automated high-performance TLC (HPTLC), which is gaining popularity, and high-performance liquid chromatography (HPLC) which is combined with mass spectrometry (MS) [13]. In addition to chromatographic techniques, vibrational spectroscopic methods, such as near-infrared (NIR), mid-infrared (MIR), and Fourier transform infrared (FT-IR), have been developed in the field of phytomedicine and are also used for QC of medicinal plants [14].

HPTLC is a widely accessible semi-automated technique used for quick sample screening to identify related herbal species and authenticate herbal products. One of the key benefits of HPTLC is its flexibility to optimize operational parameters, including a sample application, plate creation, documentation, and derivatization, [15]. Fourier transform infrared (FTIR) spectroscopy has been a valuable analytical tool in the phytomedicine field for the past few decades. It is a quick and simple technique that can be used to evaluate the plants, safety, authenticity, traceability, and adulteration on a qualitative or quantitative basis [16]. Furthermore, the technique has proven to be a quicker and less expensive alternative to the more precise but labor-intensive, time-consuming, and costly spectroscopy-based method such as NIR [17].

In recent years, chemometric modeling has made it possible to evaluate FT-IR data statistically [18]. Chemometric analysis is used to develop both qualitative and quantitative calibration models since the FT-IR spectra acquired from plant material are complicated and represent a mixture of all the functional groups present in the sample. For qualitative applications, both supervised and unsupervised models can be employed; the most unsupervised popular method is principal component analysis (PCA)[19] and orthogonal projection to latent structures-discriminant analysis (OPLS-DA) is a supervised technique. Moreover, principal component regression (PCR) and partial least square (PLS) regression are two supervised methods used to create quantitative regression models [18].

The potential for using FTIR in conjunction with chemometrics for quality control has been documented in several investigations. Using FTIR in conjunction with chemometrics, chemical components of *Centella asiatica* L collected from four different locations in Indonesia were distinguished, allowing for the identification of intra- and inter-population variations within the species [24]. Using chemometrics and FTIR data, the botanical authenticity and quality of cold-pressed functional oils sold in Romania were

determined in a different study. The developed models demonstrated a substantial ability to distinguish between the various botanical groups of oils as well as the variations across samples [23]. Correlating marker compounds with biological activity is one of the objectives of QC. A crucial step in the creation of herbal remedies is the identification of biomarkers. A product's effectiveness can be determined by looking for molecules linked to biological activities, in addition to their usage in confirming the legitimacy of plant material.

MetaboAnalyst 5.0 (https://www.metaboanalyst.ca/) is a sophisticated data processing tool, and web-based software that supports a wide range of input data, including FT-IR peak lists, NMR, and MS data. The software provides a number of chemometric options for data processing including heat maps, Random Forest, PCA, and OPLS-DA as multivariate statistical analysis; Moreover, it has some options for univariate analysis such as ANOVA and also more advanced statistical methods such as machine learning techniques [20]. This study aims to investigate the phytochemical variation of constituents produced by *S. longipedunculata*, identify marker compounds using HPTLC, and employ FTIR based chemometrics approach for the precise characterization and authentication of *Securidaca longipenduculata* Fresen. The spectra of different samples are illustrated in a cluster plot using this multivariate technique which helps the researchers detect the differences or similarities between samples.

EXPERIMENTAL

Plant Material and Extraction

Samples were obtained from Mr. Gavhi Mulatedzi, a plant harvester in Venda (Thohoyandou), who specified that the material had been collected from various locations in the Vhembe district of Limpopo Province, including Ha-Lambani, Niani, Matiyane, Muswodi, Ngwenani, Shakadza and Vhurivhuri. The identities were confirmed by Dr. Bronwyn Egan (Department of Biodiversity, University of Limpopo). Reference samples of the species are kept at the Department of Pharmacy at the University of Limpopo. An ultrasonic bath (ULTRA-SONIC, LIBMB, Labcon, Krugersdorp, South Africa) was used to extract 0.50 g of each powdered dried material for 10 min with 10.0 ml of methanol, and the extract was then filtered through a 0.45 m nylon syringe filter (Acrosdisc®, Pall, New York, NY, USA) before being transferred to individual autosampler vials for HPTLC analysis.

Chemicals and Solvents

The organic solvents used for extraction and the mobile phase were of analytical grade. Methanol (99.9% w/w), Chloroform (99.8% w/w), Ethyl acetate (99.9% w/w), Glacial acetic acid (99.5% w/w), concentrated formic acid (85.0% w/w) and sulfuric acid (95.0% w/w) were purchased from Prestige Laboratory Supplies (South Africa). *P*-anisaldehyde, a visualization reagent, was bought from Sigma Aldrich (Germany).

Analysis of Root Samples Using HPTLC

Each of the Root methanol extracts was applied in a 2 µl volume at a concentration of 50 mg ml^{-1} to a Silica gel 60 F_{254} (20 × 10 cm) glass plate from Merck Ltd. in Germany. The optimal development solvent was chloroform, ethyl acetate, methanol, and formic acid (90:10:20:1). 25 ml of the solvent was used to saturate the chamber for 20 min at 33% relative humidity and 23 ± 2 °C. After the tank was saturated, 10 ml of the mobile phase was used to allow the mobile phase to migrate over a distance of 70 mm. The plate was automatically dried in the developing chamber for six minutes following development, and then viewed under different UV radiation wavelengths (254 nm and 366 nm) and white light using an automated visualizer. After development, the plate was immersed in *P*-anisaldehyde for 2 s at a uniform vertical speed level of three (3) using a chromatogram Immersion Device, which was followed by heating the plate for 3 min at 100 °C on a plate heater to visualize the compounds. The plate was then allowed to cool for three minutes at room temperature. The main compounds that this study was focusing on were detectable at 366 nm.

FTIR Analysis of Root Samples

The root plant parts of *S. longipedunculata* samples were ground and sieved to obtain powders with consistently-sized particles. The FT-IR spectra of the samples were obtained using an Alpha-P Bruker spectrometer, mounted with an ATR diamond crystal (Bruker OPTIK GmbH, Ettlingen, Germany). OPUS 6.5 software was used to acquire the

spectral data. The procedure involves placing the powders directly on the surface of the ATR diamond crystal and pressing the plunger onto the powder to ensure direct contact with the crystal. The spectral data from 500-4500 cm^{-1} with a spectral resolution of 4 cm^{-1} were acquired in the absorbance mode. Each spectrum was taken as the average of 32 scans. The analysis was done in triplicate and the spectra were averaged using MS Excel®. Chemometric analysis of the FT-IR data was performed using MetaboAnalyst 5.0 after importing the Microsoft Excel data into the software.

Chemometrics Analysis of FTIR Data Using MetaboAnalyst 5.0

After FTIR analysis, MetaboAnalyst 5.0 (https://www.metaboanalyst.ca/) an online high-throughput metabolomic software was used to establish the relationships between the samples and variables. The Quantile normalization was conducted on the data, and then the normalized data was analyzed by two unsupervised approaches including PCA and HCA to determine the chemical variation found in *Securadaca longipedunculata* roots that were collected from seven distinct locations in the South African province of Limpopo, Vhembe region.

RESULTS AND DISCUSSION

Chemical Fingerprinting for the Identification of Chemical Markers and Inter- and Intra-Population Chemical Variation of the Root Samples

This study was the first to focus on developing an HPTLC method to identify the chemical fingerprint of *S. longipedunculata* and investigate the species variations in the chemical composition across several localities. A chemical profile is necessary to help with identification and quality control, as well as to look into differences in the chemical composition of samples taken in different places. Figure 1 displays the HPTLC profiles of *S. longipedunculata* collected from seven different localities in Venda. In this figure, Tracks 1-7 represent seven spotted samples on the plate. After repeatedly analyzing randomly chosen extracts using a variety of developing solvents and applying a variety of derivatizing agents, the optimal developing system to aid in the identification and quality control of Violet tree was discovered to be chloroform: ethyl acetate: methanol: formic acid (90:5:30:1). *P*-anisaldehyde/sulfuric acid was used as the derivatizing reagent since it produced illuminating fingerprints. This reagent helped to visualize xanthones, flavonoids, terpenes, coumarins, and steroid compounds clearly. The HPTLC plate was visualized at three distinct UV radiation wavelengths (254 nm and 366 nm) and white light, in order to determine the ideal wavelength for determining specific bands that belong to the Violet tree. In contrast to white light and UV radiation at 254 nm, the application of UV radiation at 366 nm was found to be the most effective for visualizing the species compounds. Following derivatization with this reagent, these compounds exhibit distinctive dark blue and blue fluorescent bands.

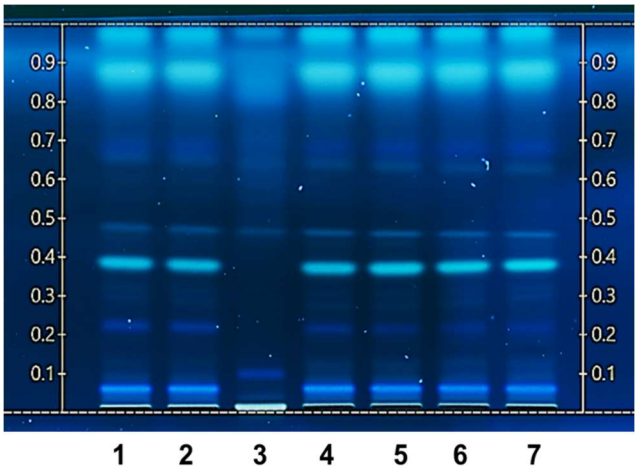

Fig. 1. HPTLC chromatogram showing chemical fingerprints of the methanol extracts of *S. longipedunculata* samples from 7 different localities (Tracks 1-7) (1 = Ha-Lambani, 2 = Niani, 3 = Matiyane, 4 = Muswodi, 5 = Ngwenani, 6 = Shakadza and 7 = Vhurivhuri. The plate was developed with chloroform: ethyl acetate: methanol: formic acid (90:5:30:1) and subsequently viewed under 366 nm reflectance after derivatization with *p*-anisaldehyde/sulfuric acid reagent.

Samples from different populations displayed some quantitative and qualitative variation in their chemistry. This was evident from the presence or absence and the size or intensity of bands when the plate was viewed under UV light of 366 nm wavelength. Qualitative differences were evident amongst sample 3 representing the Matiyane population. The

sample exhibited a dark blue band at R*f* = 0.11, and it additionally lacked the blue fluorescent bands that were present in all other samples at R*f* = 0.06 and 0.39. Quantitative rather than qualitative variation was observed for samples 4-7, representing the Muswodi, Ngwenan, Shakadza, and Vhurivhuri populations. The samples in Tracks 4-7 all contain the dark blue band at R*f* = 0.22, but their concentrations appeared to be very low. In contrast, the Matiyane sample (Track 3) appears to have low intensity of the grey band at R*f* = 0.63, dark blue band at R*f* = 0.69, and blue fluorescent band intensity at Rf = 0.99. The results indicated little variation between populations and the observed variation was attributed to geographical origin. The chemical marker compounds were selected based on their presence in most of the samples, listed in Table 1. These markers can be of assistance for identification purposes, for example, Marker 6 was identified as dark blue whose intensity correlates with its concentration. Overall, this fingerprinting method developed for *S. longipedunculata* can be used for species identification.

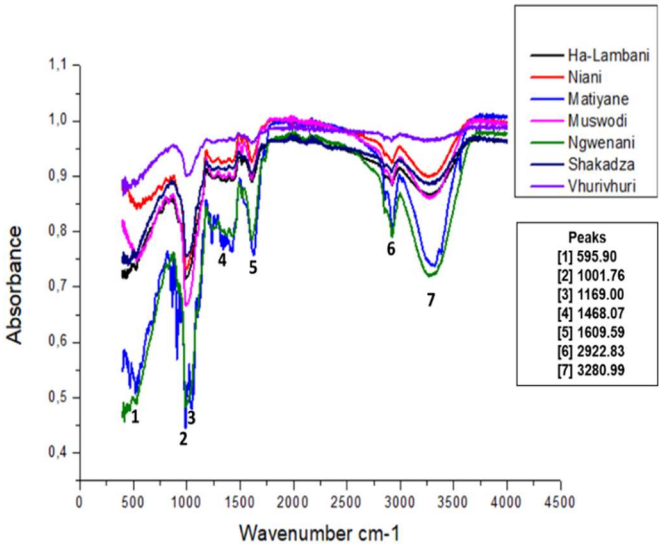

Fig. 2. FTIR spectra *S. longipedunculata* samples from 7 different localities: Ha-Lambani, Niani, Matiyane, Muswodi, Ngwenani, Shakadza, and Vhurivhuri.

FTIR Spectral Analysis of the Root Samples

The complex composition of *S. longipedunculata* roots is indicated by the broad range of functional groups observed in the FT-IR spectrum (500-4500 cm^{-1}) shown in Fig. 2. According to a review [4], flavonoids, alkaloids, saponins, tannins, and other minor chemicals are the primary chemical elements of *S. longipedunculata* isolated and identified from the roots by numerous researchers. Due to the synergistic effect of several different herb ingredients, each of these compounds plays an essential part in the complex system of contributing to the efficacy and potency of the plant. All the spectra of the species at different localities showed similar profiles with different absorbance readings. The assignment of each wavenumber to the respective functional groups is listed in Table 2.

A wide band of absorption between 3200 and 3600 cm^{-1} indicates O-H stretching vibrations, which may indicate the presence of Phenolic acids or saponins that are frequently present in plant extracts. The presence of a narrow and sharp peak at 2933-2922 cm^{-1} was assigned to show N–H stretching vibrations typical of alkaloids, while a peak at 2922.83 cm^{-1} indicated the presence of aliphatic compound by the

Table 1. List of Selected Marker Compounds in *S. longipedunculata* Methanol Extract Determined by HPTLC

Track number	Rf value	Band colour	Compound name
Track numbers 1-2, 4-7	0.06	Blue fluorescent	Unknown
Track numbers 1-2, 4-7	0.22	Dark blue	Unknown
Track numbers 1-2, 4-7	0.39	Blue fluorescent	Unknown
Track numbers 1-7	0.48	Blue fluorescent	Unknown
Track numbers 1-7	0.88	Blue fluorescent	Unknown
Track numbers 1-7	0.99	Blue fluorescent	Unknown

Table 2. Assignment of Infrared Absorption Bands for the Studied Samples

Range (cm^{-1})	Band assignment	Mode of vibration
4000-3500	The hydroxyl group (O-H) from phenols, Saponins, and Triterpenoids	Stretching
3000-2800	Alkyl groups (CH2 and CH3) from alkanes and aliphatic compounds; Amine group (N–H) from alkaloids	Stretching
1700-1600	Carbonyl groups (C=O) from ketones and aldehydes; C=C from conjugated double bonds	Stretching
1500-1400	C=C-C from aromatic rings	Stretching
1540-1175	C-O-C from ethers; C-N from amines, possibly present in alkaloids	Stretching
1175-900	O-H from phenols and C-O from primary alcohol	Bending
900-750	C−H from aromatic rings	Bending

stretching of CH_2 and CH_3 [21]. Moreover, the presence of carbonyl groups is indicated by the unique C=O stretching vibration that appears at 1600-1760 cm^{-1}, which is typical with flavonoids [22]. The stretching vibrations of C=C alkenes were correlated with the peak at 1609.59 cm^{-1}. Bands at 1500-1400 cm^{-1} could be attributed to the C=C-C aromatic ring [22]. Furthermore, the C-O-C stretching vibrations in the 1468.07 cm^{-1} region provide additional evidence for the presence of flavonoids and 1169.00 cm^{-1} related to C-N from stretching vibrations of amines [23]. Peaks in the O-H stretching area and the presence of C-O stretching vibrations within the 1000-1300 cm^{-1} range strongly imply the presence of glycosidic connections, which are saponin-specific features [23]. The presence of a narrow and sharp peak at 600-900 cm^{-1} was assigned to C-H (methoxy compounds) out-of-plane bending vibrations respectively [21].

The distinctive absorption bands do not significantly differ when viewed visually, but some wavelength intensities do vary, particularly in the fingerprint range 500-3500 cm^{-1}. This explains the similarities between a few primary chemical components found in samples of *S. longipedunculata* from various origins. Since differences in spectra are typically undetectable to the naked eye, it is more practical to use statistical methods to interpret the spectroscopic data and make it possible to distinguish the geographical origins of plant material based on small variations in specific absorption bands. Indeed, we propose employing chemometric analysis as a more reliable method for identifying the origin of samples.

Chemometrics Analysis of Root Samples Based on Metaboanalyst 5.0

The variation in chemical composition observed from the FTIR absorbance data was confirmed by the scores plot derived from a two-principal component (PC) scores plot. The PCA model was characterized by a separation of 74.7% along PC1 and 17.0% separation along PC2, as indicated in Fig. 3. The scores plot was colored according to the seven localities. The scores plot clearly shows the presence of two chemical groupings within the samples (indicated by yellow and red colored rings), although several samples are randomly distributed between the groups. The two groupings show clustering according to how close the geographical locations of the collection are to one another. For the Shakaza, Niani, and Muswodi samples, tight clustering of regions is evident; these samples are positioned along the positive PC1, suggesting that their chemical contents are comparable. The samples from Ngwenani and Matiyane exhibit a distinct separation along the negative PC2, this separation may be attributed to the higher intensity absorbance of all functional groups found at wavelengths between 500 and 3500 cm^{-1}. The samples from Ha-Lambani and Vhurivhuri showed a distinct separation, with the former clustering along the negative PC1 and the latter along the positive PC2. This separation may have been caused by the lower intensity of the stretching vibrations of the O-H (3280.99 cm^{-1}), CH_2 (2922.83 cm^{-1}), and C-O functional groups in the 1001.76 cm^{-1} region, as well as some other functional groups that exhibited weaker bands in comparison

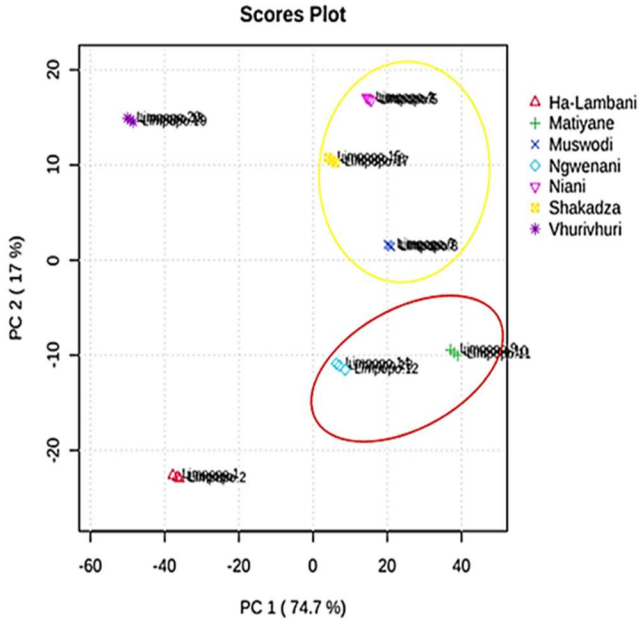

Fig. 3. Principal component analysis (PCA) scores scatter plot derived from FTIR data from different localities in Limpopo.

To samples from other localities. Clustering based on proximity to the origin area is seen along the positive PC1, containing samples from Shakaza, Niani, and Muswodi, and along the negative PC2, containing samples from Ngwenani and Matiyane. This shows that samples from nearby geographic regions have comparable amounts of the same chemical ingredients.

To evaluate patterns of similarity within the composition of the root samples, HCA analysis was conducted on the FT-IR data. As seen by the two branches of the dendrogram (Fig. 4A), the dendrogram revealed two distinct clusters. Different groups of *S. longipedunculata* primary metabolites harvested at different locations were shown in the heatmap (Fig. 4B), suggesting that metabolome patterns vary depending on the location of origin. Two primary clusters can be seen in the heatmap, with a second cluster that is further fragmented into two. *Securidaca longipedunculata* roots collected from Ha-Lambani and Vhurivhuri were grouped in the first cluster on the left wing. The second cluster was split into two groups, with Ngwenani and Matiyane gathered together while Shakaza, Niani, and Muswodi clustered and dominated the sub-cluster on the right.

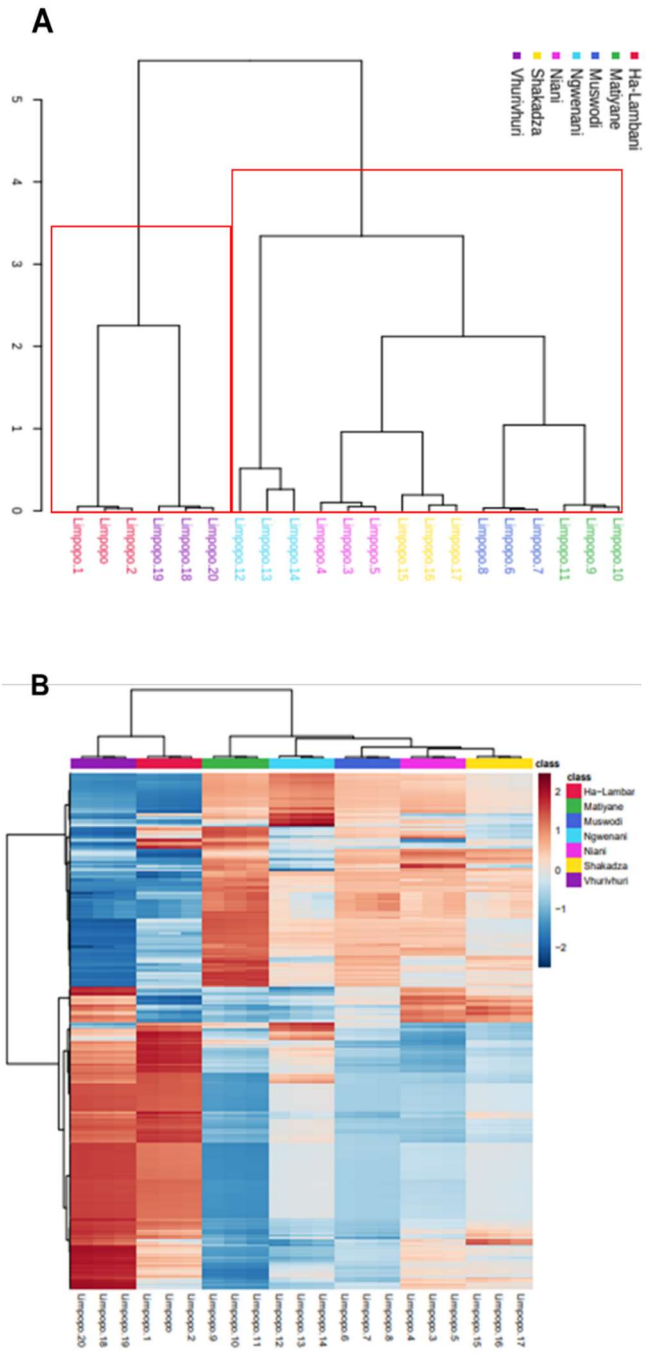

Fig. 4. (A) dendrogram derived from the FTIR analysis of *S. longipedunculata* samples from the same and different populations, indicating two chemically distinct groups, (B) Heatmap analysis representing the functional group from *S. longipedunculata* roots. The color scale shows the relative abundance of each functional group. Each row represents the functional group and each column represents a sample.

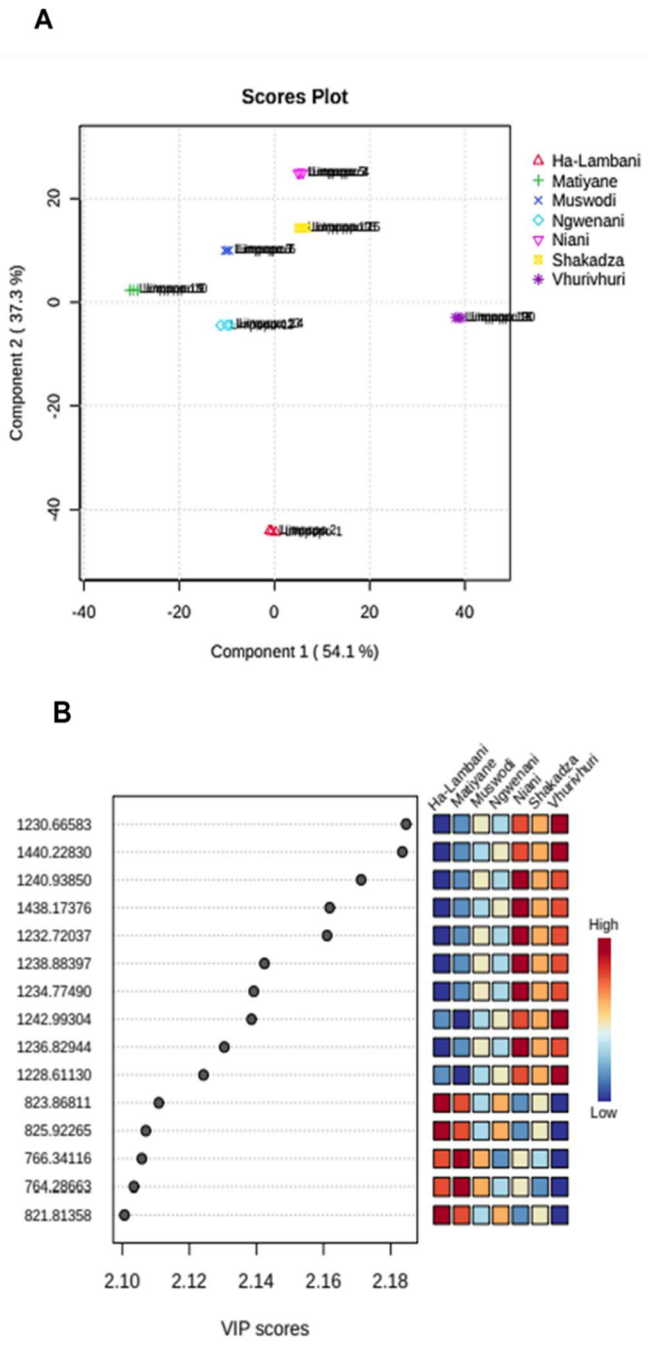

Fig. 5. (A) Score plot of orthogonal projection to latent structures-discriminant analysis (OPLS-DA) from the metabolite profile of *S. longipedunculata* roots collected in different localities, (B) The score of VIP of metabolites in partial least squares discriminant analysis (OPLS-DA) from the metabolite profile of *S. Longipedunculata* roots collected in different localities.

The HCA was performed using the PCA model to investigate the patterns further and identify the distinct groupings that were evident on the PCA scores plot. As seen on the PCA scores plot, the dendrogram (Fig. 4A) showed the existence of two major groupings (branches) with the same clustering structure. The individual samples that cluster together make it evident that the chemistry of the samples within the closeness of the location is conserved.

The OPLS-DA was utilized to find the functional groups with the highest ranking utilizing the Variable Importance in Projection (VIP) value parameters. The capacity to distinguish between samples taken from the seven distinct locations was shown by a total of 15 metabolites from the roots of the *S. longipedunculata* FT-IR spectrum. Therefore, in order to help distinguish *S. longipedunculata* samples based on their geographic origin, those 15 metabolites may be employed as potential markers in those samples. According to our research, there are differences in the metabolite profiles of the *S. longipedunculata* plant material that was gathered from Ha-Lambani, Niani, Matiyane, Muswodi, Ngwenani, Shakadza, and Vhurivhuri. This can be attributed to several reasons such as climate, soil conditions, and genetic makeup.

ACKNOWLEDGMENTS

The authors would like to acknowledge Mr. Gavhi Mulatedzi for assistance with plant collection and Dr. Bronwyn Egan for plant identity confirmation

CONCLUSIONS

High-performance thin-layer fingerprints were developed for the root bark sections of *S. longipedunculata*. The most informative profile of the root samples was produced by methanol, which was determined to be the optimal extraction solvent. Chloroform: ethyl acetate: methanol: formic acid (90:5:30:1) was the mobile phase solution that yielded the best separation, and the best derivatizing reagent was *p*-anisaldehyde/sulphuric acid. Six chemical makers could be clearly identified thanks to the ability of the established HPTLC approach for *S. longipedunculata* to resolve the chemical constituents from the root crude material. The developed HPTLC method for *S. longipedunculata* was able

to separate the chemical constituents from the root crude material to enable clear identification of six chemical markers. FTIR spectroscopy is a well-established analytical technique for rapid, high-throughput, non-destructive analysis of a wide range of medicinal plants, able to detect botanical origin. The chemical variance within the root samples was investigated using a combination of FT-IR and chemometrics. Samples from locations in the same proximity grouped closely. The analysis confirmed the quantitative differences between the samples and can be utilized to separate the root bark samples based on chemical marker content.

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