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Metabolites profiling of five *Eucalyptus* species by gas chromatography-mass spectrometry and multivariate analysis

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Abstract

An untargeted metabolomic analysis of five *Eucalyptus* species was used to compare their chemical profiles. Gas chromatography-mass spectrometry (GC-MS) along with multivariate analyses including principal component analysis (PCA) and hierarchical cluster analysis (HCA) were applied to assess variations in the leaf chemical profiles of *E. maidenii* F. Muell, *E. robusta* Smith, *E. citriodora* Hook (syn *Corymbia citriodora*), *E. tereticornis* Smith and *E. camaldulensis* Dehnh. In total, 299 relevant metabolites were identified and subjected to statistical analyses in order to detect either the major common metabolites of the genus or the specific compounds contributing to the characteristic chemical composition of each species. Organic acids, nitrogenous compounds, and phenolic constituents were the main discriminatory groups between the investigated eucalypts. The cluster analysis revealed that fatty acids and fatty alcohols were more concentrated in the leaves of *E. citriodora* and distinguished it from other species, whereas sugars, polyols, and glycosides were characteristic to *E. camaldulensis*.

Keywords: *Eucalyptus*, gas chromatography-mass spectrometry, hierarchical cluster, metabolite profiling, multivariate analysis, principal component analysis.

Introduction

Eucalyptus represents an important genus of the *Myrtaceae* family with about 800 species and varieties that are native to Australia and Tasmania (Mubarak et al., 2015). Most members of this genus are exploited for various purposes, mainly as being natural sources extensively used for timbers, pulp and essential oils (Ben Ghnaya et al., 2016). Also, *Eucalyptus* has a wide range of non-volatile compounds (Huang et al., 2015; Sebei et al., 2015; Becker et al., 2023). Due to richness of active metabolites, *Eucalyptus* has been verified for its various effective biological activities and economical values (Nasr et al., 2019a; Elangovan and Mudgil 2023; Rajapaksha et al., 2023).

Metabolomics approach is becoming a recent trend to be implemented for drug sighting, food science, and systematical biology because it can be employed to inspect molecules which are typically involved in either primary or secondary plant metabolism (Cevallos-Cevallos et al., 2009; Lavergne et al., 2018). However, no inclusive extraction technique, with high reproducibility

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Abbreviations: GC-MS - gas chromatography-mass spectrometry; HCA - hierarchical cluster analysis; IMs - individual metabolites; MGs - metabolites groups; PCA - principal component analysis.
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and robustness, is yet applicable for the recovery of all compound classes. Thus, for a complete detection of different metabolites, extraction methods may be used in combination (Kim et al., 2011).

Due to its aptitude to detect intricate metabolites mixtures with high competence and relatively low cost, gas chromatography-mass spectrometry (GC-MS) is one of the most popular techniques (Tugizimana et al., 2013). Principal component analysis (PCA) is a practical tool to observe differences between samples and the alliance or independence of the variables (Lazar et al., 2015). Subsequently, the hierarchical clustering analysis (HCA) is applied to group the variables in homogeneous clusters (Rodrigues et al., 2018).

After literature survey, we found that some previous reports have adopted metabolomics approach to analyze *Eucalyptus* extracts, especially in terms of chemical alterations as expected to be resulting from the responses of plant to a range of environmental stresses. An untargeted GC-MS metabolite profiling was adopted to outline the essential oils from two *Eucalyptus* species (Wong et al., 2017). In addition, Merchant et al. (2006a) have used the targeted metabolite profiling and revealed a quantitative discrete phytochemical relationship among eucalypts taxonomy. While, Warren et al. (2012) used GC-MS metabolite profiling to examine how the leaf metabolites would response to long and severe water stress in two species of *Eucalyptus*. It is also noteworthy that a former unbiased GC-MS metabolomic profiling in *Eucalyptus* revealed a drought stress-alterations response by several leaf metabolites with a higher number of distorted compounds than of targeted analysis (Fraire-Velázquez and Balderas-Hernández, 2013). In two recent studies, Correia et al. (2018) have used both GC-MS and principal component analyses (PCA) to assess the combined effects of some ecological stresses on the protective responses of *E. globolus*, while, Mokochinski et al. (2018) have used a combined approach of GC-MS and LC-MS for the analyses of polar and semi-polar compounds in some *Eucalyptus* extracts *via* both non-targeted and targeted analyses. Lastly, Bragunde et al. (2023) have used GC-MS to analyze the leaf metabolome of four *Eucalyptus* species to assess their polar and non-polar chemical componunds for feeding preferences. The current study aimed to investigate and compare the chemical composition of *E. maidenii*, *E. robusta*, *E. citriodora* (syn *Corymbia citriodora*), *E. tereticornis,* and *E. camaldulensis* species *via* GC-MS profiling with a subsequent multivariate analysis, highlighting main groups of primary constituents as well as some secondary metabolites in the plant leaves.

Materials and methods

Plant samples: Full-grown leaves of five *Eucalyptus* species (fruiting stage, the trees were roughly estimated to be aged around 60 - 70 years) were collected from botanical garden, Kunming Institute of Botany, Chinese Academy of Sciences, Heilongtan, Kuming, Yunnan, China. The plants were subjected to the regular climatic conditions of the area without any fertilization effect. The plant samples were collected in a randomized complete block design from 3 - 4 trees per each species (five leaves from each tree). The five species were authenticated and voucher samples were preserved in the Department of Biosciences, College of Life Sciences, Anhui Normal University [*E. camaldulensis* (CLS-Eu101), *E. maidenii* (CLS-Eu102), *E. robusta* (CLS-Eu103), *E. citriodora* (syn *Corymbia citriodora*, CLS-Eu104), *E. tereticornis* (CLS-Eu105)].

Instruments: Automatic sample rapid grinding instrument *JXFSTPRP-24/32* (*Shanghai Jingxin Industrial Development Limited Company*), ultrasonic cleaning machine *SB-5200DT* (*Ningbo Xinzhi Biotechnology Co., Ltd.*), whirlpool oscillator *TYXH-I* (*Shanghai Hannover Instrument Limited Company*), desktop high-speed refrigerated centrifuge *TGL-16MS* (*Shanghai Luxiangyi Centrifuge Instrument Limited Company*), freeze concentration centrifugal direct fired dryer *LNG-T98* (*Taicang Huamei Biochemical Instrument Factory*), gas bath constant temperature oscillator *THZ-82A* (Jintan City, *Jiangsu Huanyu Scientific Instrument Factory*), vacuum drying box *DZF-6021* (*Shanghai Huitai Instrument Manufacturing Limited Company*), GC-MS *7890 B-5977A* (*Agilent Technologies Limited*), color spectrum column *DB-5MS* (30 m × 0.25 mm × 0.25 µm) (*Agilent*).

Reagents: All solvents and chemicals used for GC-MS analysis were of analytical or HPLC grade: methanol, pyridine, *n*-hexane, methoxylamine hydrochloride (97%), and N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) with 1% trimethychlorosilane (TMCS) were purchased from *CNW Technologies* (Düsseldorf, Germany). Trichloromethane was obtained from *Sinopharm Chemical Reagent Co.* (Shanghai, China), and L-2-chlorophenylalanine was ordered from *Shanghai Hengchuang Bio-technology Co.* (Shanghai, China).

Extraction of plant samples: *Eucalyptus* leaves (three replicates were considered for each sample) were extracted as follows; a leaf powder (30 mg) was mixed with 360 µL of a precooled methanol and 40 µL of 2-chloro-l-phenylalanine (0.3 mg/mL, dissolved in methanol, was added as an internal standard) for the extraction process. Samples were incubated at -80°C for 2 min, and then grounded at 60 Hz for 2 min. The resultant mixture was first sonicated and then, 200 µL of chloroform was added to the samples with speedy vortexing. 400 µL water was added and vortexed again, then ultra-sonicated at ambient temperature for 30 min. After centrifugation at 14 800 *g* for 10 min at 4°C, an aliquot of 500 µL supernatant was transferred to a glass sampling vial and dehydrated at room temperature, followed by re-dissolving in 80 µL of 15 mg/mL methoxylamine hydrochloride in pyridine. The mix was vigorously vortexed for 2 min prior to incubation at 37°C for 90 min. Afterward, 80 µL of BSTFA with 1% TMCS and 20 µL *n*-hexane were mixed with the previous solution and combined by shaking for 2 min using a vortex with maximum speed. The derivatization of

samples was carried out at 70°C for 60 min. A centrifugation step was run at 14 800 *g* for 5 min to remove any formed precipitate, which was then isolated from supernatant. The latter was reserved at ambient temperature for 30 min prior to GC-MS analysis.

Chromatographic conditions and GC-MS analysis: The five species of *Eucalyptus* were investigated by GC-MS *via* a fingerprinting mode to compare the leaf chemical profiles. For the separation and profiling of metabolites (derivatized samples), data acquisition was carried out using an *Agilent 7890B* gas chromatography system coupled to an *Agilent 5977A MSD* system (*Agilent Technologies*, Santa Clara, CA, USA) and a *DB-5MS* fused-silica capillary column (30 m × 0.25 mm × 0.25 µm; *Agilent J & W Scientific*, Folsom, CA, USA). Helium (> 99.999%) was used as the carrier gas under a constant flow rate of 1 mL/min. The injector temperature was maintained at 260°C. Injection volume was 1 µL by splitless mode. The initial oven temperature was 60°C, ramped to 125°C at a rate of 8°C/min, to 210°C at a rate of 5°C/min, to 270°C at a rate of 10°C/min, to 305°C at a rate of 20°C/min, and finally held at 305°C for 5 min. The temperatures of MS quadruple and ion source (electron impact) were set to 150 and 230°C, respectively. The collision energy was 70 eV. Mass spectrometric data were acquired in a full-scan mode (*m/z* 50-500), and the solvent delay time was set to 5 min. The QCs were injected at regular intervals (every 10 samples) throughout the analytical run to provide a set of data from which repeatability could be assessed.

Data processing: *ChemStation* (*version E.02.02.1431*, *Agilent*) software was used to convert the raw data (D format) to CDF format, and then the CDF data were imported into the *ChromaTOF* software (*version 4.34*, *LECO*, St. Joseph, MI, USA) for data processing. Metabolites were annotated through *Fiehn* or *NIST* database. After alignment with *Statistic Compare* component, the 'raw data array' (cvs) was obtained from raw data with three-dimension data sets including sample information, peak names (or retention time and *m/z*) and peak intensities. There were 1 020 peaks detected from all samples which were next filtered. In the 'data array', all internal standards and any known pseudo-positive peaks (caused by background noise, column bleed or BSTFA derivatization procedure) were removed. The data were normalized to the total peak area of each sample, and multiplied by 10 000, and the peaks from the same metabolite were combined. The total detectable compounds were 326. The compounds with retention indexes (RI) and/or mass spectra that did not match well with the available GC-MS databases, even though they were automatically annotated by the GC data processing, were ultimately excluded. The consequential database, with the relative intensities of annotated metabolites for each sample, was integrated for following statistical analysis.

Statistical analysis: Data were transformed by 10log in *Excel 2007* (*Microsoft*, USA) (use 0.000001 to replace 0 before transforming) then, scaled by mean-centering and division by the standard deviation square root of each variable, the statistically significant differences were compared among mean values at the level of significance ($P<0.05$) and evaluated with *ANOVA*. The resulting data matrix was then imported into *MATLAB Statistical Tool Box* (*version 9.0*, *R2014a*). Principle component analysis (PCA) was performed to visualize the metabolic difference among investigational groups, after mean centering and unit variance scaling. For hierarchical cluster analysis (HCA), data of the content value of each compound were normalized to complete linkage hierarchical clustering in order to obtain a heat map of relative differences in metabolites in the five *Eucalyptus* species. Correlation coefficients within the above-mentioned interpretations were considered according to Jolliffe (2002).

Results

Gas chromatography-mass spectrometry (GC-MS) data: The GC-MS chemical profiling exposed 299 relevant peaks, representing several plant primary metabolites in addition to a significant number of secondary compounds, collectively named as individual metabolites (IMs). The metabolites were also manually grouped into 11 metabolite groups (MGs). Both IMs and MGs were further subjected to statistical analyses in order to detect either the major common metabolites of the genus or the specific compounds contributing to the characteristic chemical composition of each species.

Individual metabolites (IMs): The first 24 copious compounds were highlighted along with the corresponding species in which they were maximally detected (Table 1 Suppl.). Myo-inositol was the highest presented with a relative abundance of (1 328.68%), recorded for *E. camaldulensis*, followed by galactinol (973.6%), quinic acid (855.03%) and tagatose (822.92%). The total individual metabolites (299) were subjected to PCA and HCA analyses, and a dendrogram was constructed to attain the association between *Eucalyptus* species.

Metabolite groups (MGs): Pertaining to their chemical structures, the resulted 11 MGs were as follows - amino acids (MG 1), fatty acids and fatty alcohols (MG 2), organic acids (MG 3), sugars, sugar acids and polyols (MG 4), phosphate and ascorbate (MG 5), nitrogen-containing compounds (MG 6), aromatic compounds (MG 7), glycosides (MG 8), terpenoids (MG 9), phenolic constituents (MG 10), along with other compounds (MG 11). Compounds belonging to each group are presented in Table 2 Suppl. MGs were also analyzed *via* PCA and HCA and a dendrogram combined with a heat-map was generated to signify the data.

Principal component analysis (PCA): A total of 10 diagrams were generated in the PCA model. They were designed based on five principal components (PCs), and wholly explained 43.38% of the average variation per species in case of IMs and 45.52% per species in case of

MGs. One PCA score-plot diagram (expressing the highest separation between species) and its parallel PCA loading diagram were selected to be displayed here. The variations in IMs were scaled by the graphical model of PC 2 *versus* PC 3, which could separate *Eucalyptus* into groups (Fig. 1). The discriminating variables, which contributed mainly to such separation, were displayed on the related loading plot (Fig. 2).

Nevertheless, the segregation between *Eucalyptus* species was better assessed *via* the PC analysis of MGs especially when PC 2 was considered against PC 4 (Fig. 3), the corresponding loading plot showing the MGs responsible for discrimination between species were shown in Fig. 4.

Hierarchical cluster analysis (HCA) and heat map: For both IMs and MGs, the cluster analysis revealed two main groups of species, but the resulted sub-groups were different in each case. HCA of IMs indicated that both *E. citriodora* and *E. tereticornis* were set together in one cluster and the second cluster combined the other three *Eucalyptus* species, an independent dendrogram (without color map) was established using the between-group linkage method (Fig. 5).

Alternatively, in Fig. 6, when MGs were used to create a dendrogram, *E. citriodora* was located in one group while other remaining four eucalypts were initially clustered together in the second group. To have a clear and general insight on the changes of metabolites across species, a heat-map was performed along with HCA, in which *E. citriodora* had a relatively maximum presence of fatty acids or alcohols (MG 2) and aromatic compounds (MG 7, high abundance) as concluded from the color scale at left side of the heat map. *E. tereticornis* was characterized

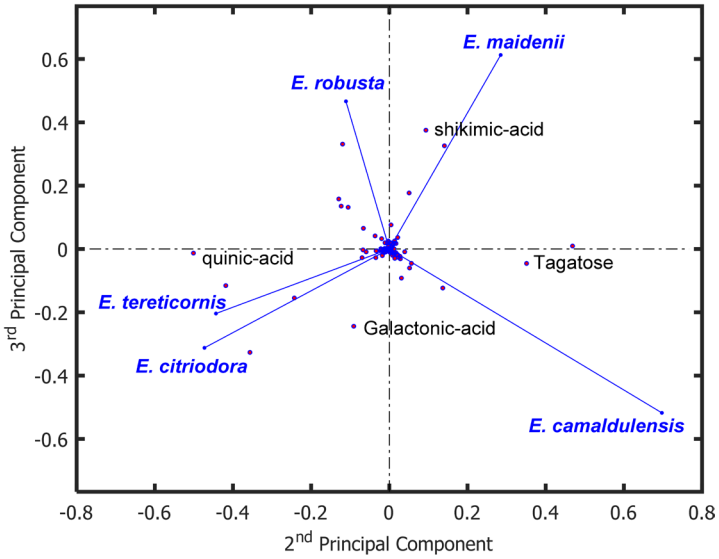

Fig. 1. A score bi-plot for scaled PC showing the relationship between *Eucalyptus* samples based on their IMs.

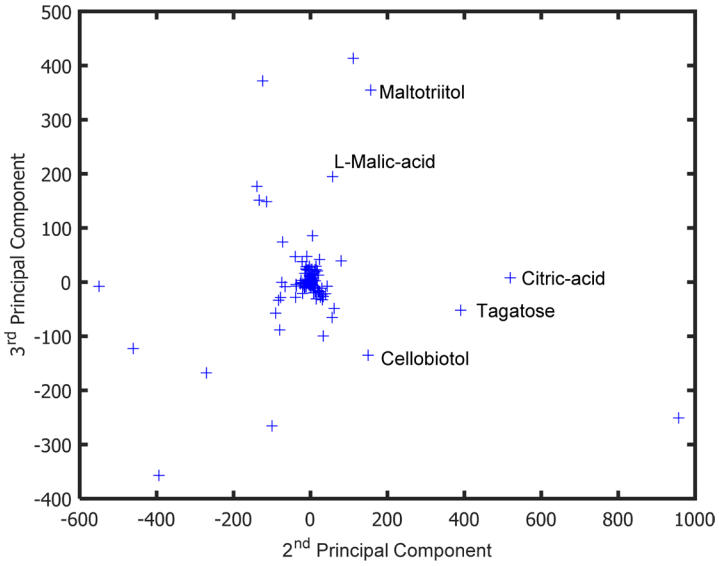

Fig. 2. Two-dimensional loading plot illustrating the IMs that are responsible for the discrimination of *Eucalyptus* samples as observed in the score bi-plot.

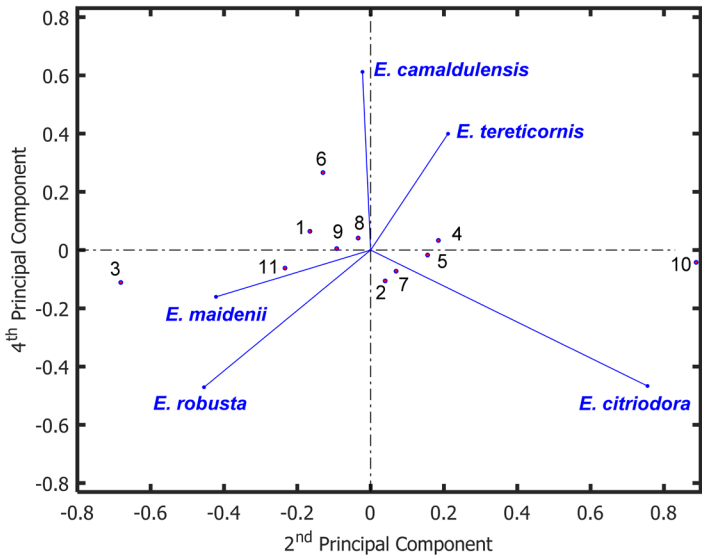

Fig. 3. A score bi-plot for scaled PC showing the relationship between *Eucalyptus* samples based on their MGs which were represented by their corresponding numbers 1, 2, *etc*.

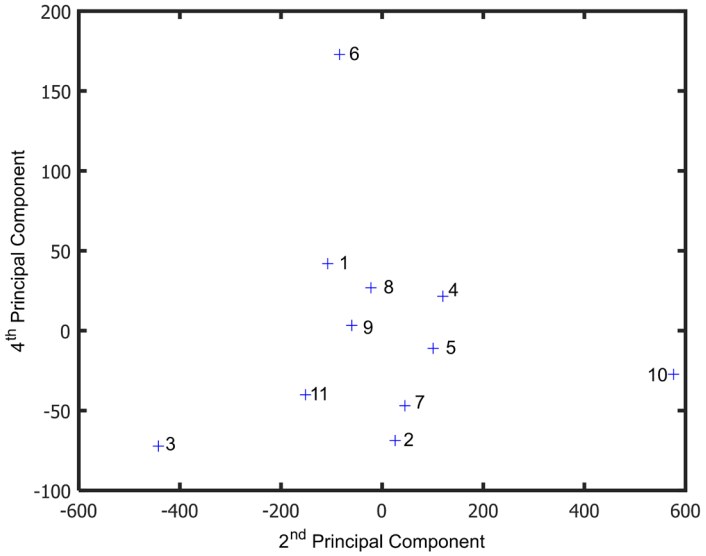

Fig. 4. Two-dimensional loading plot illustrating the MGs that contributed to the discrimination of *Eucalyptus* samples.

by the presence of nitrogen-containing compounds (MG 6, very high abundance) and high abundances of phosphate or ascorbate (MG 5) with phenolic compounds (MG 10). A noticeable high abundance of both MG 9 (terpenes and terpenoids) and MG 11 (other compounds) with a moderate presence of amino acids (MG 1) were recorded in *E. robusta*, which was mainly similar to *E. maidenii* (connected *via* one sub-sub-cluster). The latter had a moderate abundance of sugars and polyols (MG 4). Lastly, *E. camaldulensis* maintained a very high abundance of glycosides (MG 8) and a high abundance of sugars and polyols (MG 4).

Discriminating metabolites: Noticeably, maltotriitol, galactonic acid, tagatose, myo-inositol (sugars and polyols), citric acid (organic acids) in addition to some phenolic constituents including gallic and quinic acids as well as shikimic acid (antioxidant) were spotted as the discriminating molecules (IMs) between species, these metabolites were denoted on both PCA score and loading plots. While, organic acids (MG 3), nitrogen-containing compounds (MG 6) and phenolics (MG 10) were highlighted as the differential metabolite groups (MGs) between *Eucalyptus* samples which contributed to the segregation of species.

Correlation coefficients: The correlation coefficients between the five species were considered once for IMs and again for MGs as shown in Tables 1 and 2.

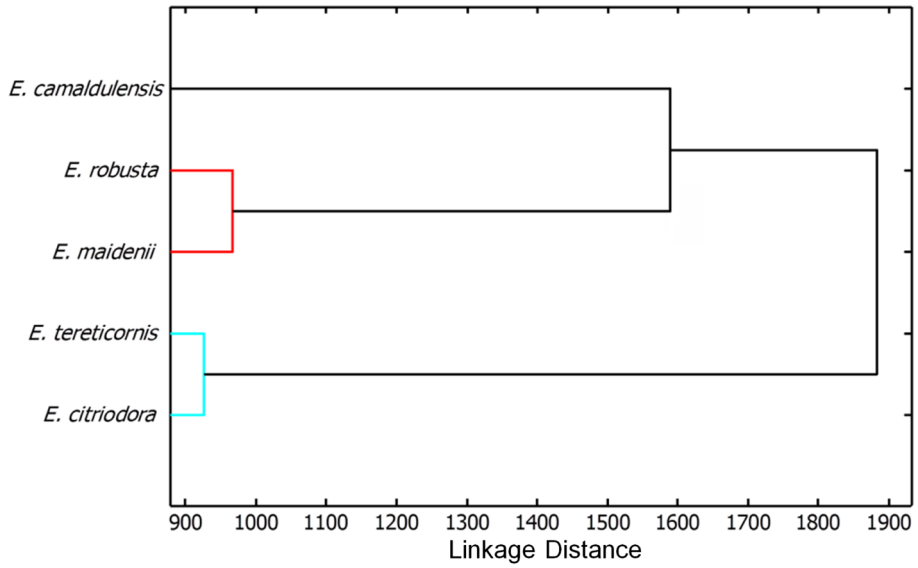

Fig. 5. A dendrogram obtained by hierarchical cluster analysis based on the Euclidean distance between leaf metabolites (IMs) of five *Eucalyptus* species.

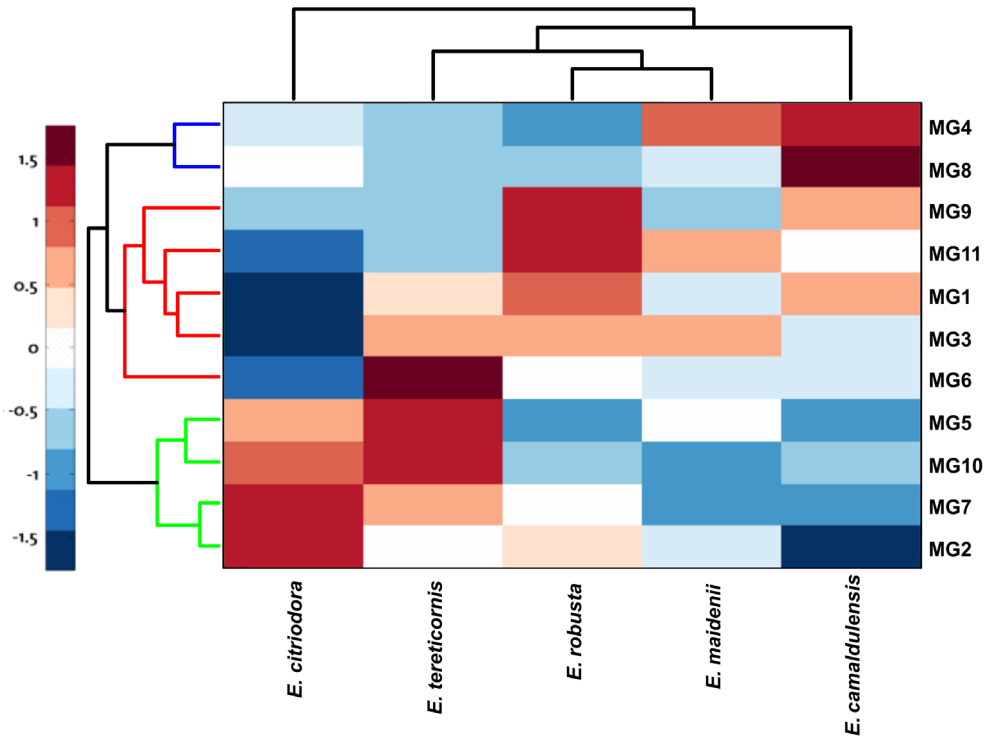

Fig. 6. A dendrogram combined with a heat-map displaying the levels of metabolite groups (MGs) in *Eucalyptus*. MG 1: amino acids, MG 2: fatty acids and fatty alcohols, MG 3: organic acids, MG 4: sugars and polyols, MG 5: phosphates and ascorbate, MG 6: nitrogenous compounds, MG 7: aromatic compounds, MG 8: glycosides, MG 9: terpenes and terpenoids, MG 10: phenolic compounds and derivatives, MG 11: other compounds. Metabolite contents per group were z-transformed, analyzed *via* hierarchical clustering, and differentiated by color design (*dark red*: high abundance, *light red*: low content).

Discussion

An exploring of metabolism is pivotal to understand the phenotypic behavior of *Eucalyptus*, which is a very prevalent genus that can grow and adapt to various habitats worldwide (Mokochinski et al., 2018). *Eucalyptus* is a large genus including both wild and hybrid species (Sharma et al., 2023). So, it is crucial to investigate

Table 1. Correlation coefficient values of the relationships between five *Eucalyptus* species based on their IMs analysis (* - correlation probability < 0.01).

Species	*E. maidenii*	*E. robusta*	*E. citriodora*	*E. tereticornis*	*E. camaldulensis*
E. maidenii	1.000	0.810*	0.535*	0.627*	0.728*
E. robusta	0.810*	1.000	0.739*	0.776*	0.589*
E. citriodora	0.535*	0.739*	1.000	0.851*	0.448*
E. tereticornis	0.627*	0.776*	0.851*	1.000	0.508*
E. camaldulensis	0.728*	0.589*	0.448*	0.508*	1.000

Table 2. Correlation coefficients of the relationships between five *Eucalyptus* species based on their MGs analysis (* - correlation probability < 0.01).

Species	*E. maidenii*	*E. robusta*	*E. citriodora*	*E. tereticornis*	*E. camaldulensis*
E. maidenii	1.000	0.995*	0.970*	0.974*	0.995*
E. robusta	0.995*	1.000	0.961*	0.983*	0.984*
E. citriodora	0.970*	0.961*	1.000	0.974*	0.983*
E. tereticornis	0.974*	0.983*	0.974*	1.000	0.971*
E. camaldulensis	0.995*	0.984*	0.983*	0.971*	1.000

the amount of plant metabolites because their qualitative or quantitative variations could give a good indication to the chemical uniqueness of each species and how it reacts towards environmental stresses (Talhaoui et al., 2015).

For a complete detection of different metabolites, the extraction process should be carried out *via* an adjusted specific/selective protocol (Kim et al., 2011). A number of studies have shown that using solvents with different polarities captured various active constituents from *Eucalyptus* (Khan et al., 2012; Kołodziejczyk et al., 2013; Lou et al., 2016; Zhang et al., 2016; Pimenta et al., 2018). Both conventional and non-conventional extraction techniques have been employed to obtain the bioactive compounds from different *Eucalyptus* species (Bhuyan et al., 2015; 2016). The currently used extraction method was first chosen based on our prior results from two studies on *Eucalyptus* (Nasr et al., 2019a,b) in which we compared different extraction solvents and methanol and yielded the highest amounts of crude extracts after either successive or selective extraction of *E. camaldulensis* leaf, bud, and capsule. This was consistent with Jaroszyńska (2003) who investigated the effect of solvent type on the losses in the crude phytoconstituents during the extraction procedure and noted that the best recovery was achieved with methanol. So it was further applied in previous investigations which adopted the GC-MS analyses to explore the chemical compounds of *Eucalyptus* species.

From the topology of the score plots, PCA analysis allowed separation of *Eucalyptus* samples into groups and showed differentiation in their chemical composition. The best separation among *Eucalyptus* species relied on the MGs analysis, when PC 2 against PC 4 could typically distinguish between *Eucalyptus* species as further went parallel with HCA clusters. These PCs are mostly influenced by organic acids and sugars.

HCA analysis was applied to see how the metabolites deviate between the species. A successful differential system could be employed here to classify the five *Eucalyptus* species into two main groups based on their metabolic profiling *via* MGs. In this context, *E. citriodora* was solely located in one group, while other four species were further subdivided into extra sub-groups within the second main group. An additional analysis of heat-map demonstrated the variations in MGs content in different *Eucalyptus* species; as it visualized that fatty acids and fatty alcohols were more concentrated in the leaves of *E. citriodora* and distinguished it from other species, whereas sugars, polyols, and glycosides were characteristic to *E. camaldulensis*, and with less abundance, terpenoids were also detected. Furthermore, *E. robusta* and *E. maidenii* were closely connected by one sub-sub group, thus, they are probably closer in their chemical characteristics. By looking back to the constructed heat map focusing on only these two species, we may suggest that these two samples had similar chemical composition in view of organic acids. Conversely, terpenes and terpenoids can be used to distinguish between *E. maidenii* and *E. robusta* due to their high abundance in the latter. Meanwhile, *E. tereticornis* was mostly characterized by the presence of nitrogenous compounds, phenolic constituents plus some phosphate and ascorbate salts. Interestingly, despite of the numerous detectable organic acids (MG 3) by GC-MS, they were equally represented with low abundance in three species (*E. tereticornis*, *E. robusta*, and *E. maidenii*) and hence, they cannot be intended for separation between eucalypts. Also, phenolic compounds were only present in *E. tereicornis* and with a relatively lower abundance in *E. citriodora*. Though, many former reports confirmed the presence of phenolic constituents in other *Eucalyptus* leaf extracts (Vuong et al., 2015; Sastya et al., 2018; Nasr et al., 2019a,b). Noteworthy, in case of such doubtful absence, it may be assumed that their concentrations were found below detection limits. However, more thorough characterization using specific

quantification techniques is still required for further confirmation.

Several polar metabolites, frequently primary metabolites, were detected. Maltotriitol, galactonic acid, tagatose, myo-inositol (sugars and polyols), citric acid, and L-malic (organic acids) in addition to some phenolic constituents were among differential individual metabolites (IMs). In accordance with the GC-MS data, Warren et al. (2012) found that shikimic acid and some common sugars such as fructose and myo-inositol were copiously present in *Eucalyptus.* Also, qualitative high abundances of some cyclitols, chiefly myo-inositol, were obviously detected in different eucalypts (Merchant et al., 2006b; Warren et al., 2012; Mokochinski et al., 2018). *Eucalyptus* is characterized by its plasticity and high capacity to survive and flourish in different habitats; this is partially noted to its ability to produce a wide range of specialized secondary metabolites in addition to essential primary compounds. Inositol (myo-inositol) has been tried for the treatment of some depression disorders and respiratory distress syndrome, in addition to its role in signaling of insulin (Taylor et al., 2004; Amaefule et al., 2018; Regidor et al., 2018; Howlett et al., 2019).

In the current study, some organic acids have contributed considerably to the discrimination between *Eucalyptus* species. Many interesting biological roles have been ascribed to organic acids, delineating their antioxidant, anticoagulant, antibacterial, anti-inflammatory, analgesic, and anti-diabetic effects (Rabelo et al., 2016; Veach et al., 2016; Al-Malki, 2019; Kim et al., 2019; Souza et al., 2019). Generally, organic acids, as an extremely common metabolite group in plants, are consistently used to reduce food-borne pathogens contamination and eliminate food spoilage in many countries (Bai et al., 2019).

It is worthy to mention that GABA is a significant compound which was found in both *E. tereticornis* and *E. maidenii*, and with a relatively less abundance, in *E. camaldulensis*. It is important for plant developmental processes including signaling, and for plant defenses mechanisms against biotic and abiotic stresses (Otto et al., 2017). Also, GABA exerts many applicable roles as a regulator compound in carcinogenesis, pain indulgence, and inflammation (Chen et al., 2018).

Added to the above, some phenolic acids including gallic, chlorogenic, and quinic, were proved to play a considerable role in *Eucalyptus* species. The therapeutic significances of gallic acid have been introduced in many earlier reports (Choubey et al., 2015; Nayeem et al., 2016; Kosuru et al., 2018; Dludla et al., 2019). Taking into account, Agunloye and Oboh (2018) have reported that chlorogenic acid can prevent peroxynitrite formation and decrease the possibility for lipid peroxidation in heart. Also, quinic acid was proved to exhibit several biological activities, with the most important as antioxidant, antimutagenic, anti-inflammatory and antimicrobial agent (Bai et al., 2019).

In view of MGs, it appears that three groups which are organic acids, nitrogenous compounds, and phenolic constituents were set as distinctive factors among *Eucalyptus* species, at least, for a general separation mean. Therefore, organic acids and phenolic compounds, as commonly highlighted for both IMs and MGs, are likely to be the most effective in the differentiation of *Eucalyptus* species *via* interpreting GC-MS data through the multivariate analyses.

Conclusions

In this work, the GC-MS chemical profiles of *Eucalyptus* species reflected an overview of the leaf metabolites and the chemical composition distinctive to each species, which has not been investigated so far. The grouped classification of metabolites (MGs) was more effective than the individual ones (IMs), because the former could give a reasonable and clear separation between species. Also, we could establish a heat map combining all 11 MGs once, which was inaccessible in case of IMs due to its large and disperse inputs. The current work would participate in distinguishing *Eucalyptus* species based on their chemical profiles. Considerable chemical constituents from five *Eucalyptus* species were highlighted, providing a general database spreadsheet for future investigations of this genus. However, due to the intrinsic limitations of GC-MS inability to measure either thermo-labile metabolites or high molecular mass polar compounds (>500 Da), overall extraction procedures and chromatographic techniques would be further applied to corroborate other secondary metabolites and hence, can offer more refined limits for accurate categorization.

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