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Metabolomic and molecular signatures of Mascarene Aloes using a multidisciplinary approach

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A B S T R A C T

In this research a multidisciplinary approach was used to unveil the genetic, metabolic uniqueness and relation-ships of endemic Mascarene Aloes (Aloe macra, Aloe purpurea, Aloe tormentorii) with respect to Aloe vera. Nuclear magnetic resonance spectroscopy, DNA sequencing and antioxidant profiles of these Aloes were studied. Principal component analysis following 1H NMR revealed the specificity of the Mascarene Aloes relative to Aloe vera. The superior free radical scavenging ability of A. purpurea, A. macra and A. tormentorii as compared to other Aloes was also unveiled. Phylogenetic analyses of chloroplast genes and ITS region sequences of these Mascarene Aloes were done using maximum parsimony and Bayesian analysis. Mascarene Aloes clustered within one clade separate from Aloe vera confirming their relative recent emergence in this genus. Results from this study showed that there is sufficient evidence at the metabolomic and molecular level to distinguish between Aloe purpurea from Mauritius and that of Reunion.

1. Introduction

The Mascarene Islands situated in the south west of the Indian Ocean comprise three countries: Reunion, Mauritius and Rodrigues and share a rich indigenous biodiversity. The use of plants to treat various ailments forms a fundamental component of the lifestyle of the Mascarene people. Aloe tormentorii (Marais) Newton and Rowley and Aloe purpurea Lam. are species endemic to Mauritius (Bosser et al., 1976; Gurib-Fakim, 2003) and are commonly known as ‘Mazambron marron’ in this country. Aloe macra Haw. is endemic to Réunion Island (Bosser et al., 1976; Pailler et al., 2000) and phenotypically resembles A. purpurea but it has slender leaves of about 70 × 10 cm and smaller inflorescences (Ranghoo-Sanmukhiya et al., 2010). The leaves of A. purpurea are traditionally used to treat cutaneous bacterial infections and boils and as well as an antispasmodic to relieve stomach pains (Gurib Fakim et al., 2003; Govinden-Soulange, 2014; Lobine et al., 2015). A. macra, A. purpurea and A. tormentorii, in spite of having morphological differences, are often confused with other Aloe species particularly with the local Mazambron (A. vera/A. barbadensis Miller) (Guého, 1988). In this study, a multidisciplinary approach was used to resolve the affinities of the Mascarene Aloes including nuclear magnetic resonance spectroscopy, DNA sequencing and antioxidant profiling.

Chase et al. (2000) were the first to investigate phylogenetic relationships in Asphodelaceae using chloroplast DNA (cpDNA) sequence data and Alooeidae were recovered as monophyletic with respect to other taxa studied (Chase et al., 2000). Recently, Grace et al. (2015) investigated the evolutionary history of Aloes and correlated leaves succulence to their medicinal use. Other phylogenetic work within the Asphodelaceae was concentrated on the subfamily Alooeidae, using cpDNA sequences and genomic fingerprinting inter simple sequence repeat markers (ISSRs) (Treytelein et al., 2003). Ramdhani et al. (2011) have elucidated the monophyly of Haworthia species using both chloroplast trnL-trnF, psbA-trnH and nuclear internal transcribed sequence one (ITS) markers.

Recent studies using chromatographic techniques have correlated the phytochemical and antimicrobial attributes of A. macra, A. purpurea and A. tormentorii and validated their use in the folk medicine of the Mascarene Islands and the anthraquinone profile of Mascarene Aloes has also been described (Ranghoo-Sanmukhiya et al., 2010) hence highlighting their prospective antioxidant attributes. Van Wyk et al. (2008) reviewed the various traditional uses of African Aloes highlighting Aloe ferox as the most renowned and widely used medicinal plant in Southern Africa. Likewise, several authors have described the antioxidant capacity of the reputed Aloe vera (Hu et al., 2003; Hu et al., 2003; Hu et al., 2003; Hu et al., 2003; Hu et al., 2003; Hu et al., 2003; Hu et al., 2003; Hu et al., 2003; Hu et al., 2003; Hu et al., 2003; Hu et al., 2003; Hu et al., 2003; Hu et al., 2003).
Saritha et al., 2010; Lee et al., 2012; Padmanabhan et al., 2012). The leaf gel of A. ferox and A. greatheadii var. davynana (birtses) have been reported to exhibit antioxidant activities (Loots et al., 2007). Dagne et al. (2000) have reviewed the chemistry and chemotaxonomy of Aloe species and described the major classes of compounds present in Aloes. However, the antioxidant attributes of Mascarene Aloes have not yet been reported.

In addition to the above methods, NMR spectroscopic profiles of the Mascarene Aloes were compared in this study. NMR spectroscopy represents a reproducible, wide-spectrum chemical analysis technique and stable in time requiring very simple material preparation (Choi et al., 2004). NMR with PCA has been applied in the metabolomic profiling of wines, juices, beers and many plant species (Choi et al., 2005). Jiao et al. (2010) have used 1H NMR spectroscopy to validate the purity of Aloe vera products and Campestrini et al. (2013) have used 13C and 1H NMR to establish the acetylation pattern of the polysaccharide, glucomannan, present in Aloe barbadensis Miller (A. vera).

The focus of this study was to determine the suitability of using both chloroplastic DNA sequences and nuclear ITS sequences for deriving the phylogeny of the Mascarene Aloe species. Additionally, the chemosystematics of Aloes from the Mascarene Islands is unveiled using a multidisciplinary approach through the integration of metabolomic and molecular data. The metabolic and genetic signatures of these Aloes are deciphered by using a chemometric approach and DNA sequences thus representing a critical method by which the Mascarene Aloes can be differentiated.

2. Materials and method

2.1. Plant material

Leaves from three year old plants of indigenous Mascarene Aloe plants were harvested from the Conservatoire Botanique National de Mascarin, Reunion Island and Mauritius Herbarium garden, MSIRI (Table 1). Samples were immediately lyophilized and ground into a fine powder. Fresh leaves were kept at −40°C for molecular work. Voucher specimens were deposited at the Mauritius Herbarium.

2.2. NMR-analysis

2.2.1. Extraction and sample preparation

Deuterated methanol CD3OD (99.9%), water D2O (99.0%) and Sodium 3-Trimethylsilylpropionate) TMSP, were purchased from Eurisotop, France. The dry powdered plant material (50.0 mg) was treated with 2.0 ml of a 70:30 mixture of CD3OD and D2O (buffered at pH 7.2) containing TMS 0.01% in 2 ml Eppendorf tube. The extract was vortexed for 1 min at room temperature and then ultrasonicated for 10 min at room temperature. The mixture was then centrifuged at 13,000 rpm for 10 min and the supernatant was transferred to 2.0 ml microtube. The mixture was centrifuged once again for 1 min at room temperature and the supernatant was then distributed into 5 mm-NMR tubes for measurements.

2.2.2. NMR measurements

1H-NMR spectra were recorded at 300 K on a Bruker Avance 500 MHz NMR spectrometer (Bruker, Karlsruhe, Germany) operating at a proton NMR frequency of 500.13 MHz for 1H, using a triple BBI probe. Deuterated methanol was used as the internal lock. 128 scans of 32 K data points with a spectral width of 10,330 Hz were recorded with the following parameters: pulse width (PW) = 30°, and relaxation delay (RD) = 1.0 s. The acquisition time was 3.17 s. FIDs were Fourier transformed with LB = 0.3 Hz. The resulting spectra were manually phased and calibrated to 0.00, using the internal standard TMS and topspin software (version 3.9, Bruker). Spectral intensities were normalised to total intensity and reduced to integrated regions of equal width (0.04 ppm) corresponding to the region of δ 4.50 – δ 5.24 and δ 3.30 – δ 3.35 were removed prior to further analysis because of the residual signals of water and methanol, respectively. The matrix size consisted then of 220 variables. Principal component analysis (PCA) was performed with AMIX (version 3.9, Bruker).

2.3. DNA isolation

Genomic DNA extraction was done using a modified CTAB protocol described by Govinden-Soulange et al. (2007).

2.4. PCR amplification

The trnH-psbA intergenic spacer, matK (matK) and Ribulose bisphosphate carboxylase large chain (rbcL) genes that reside on the chloroplast genome and the ITS internal transcribed sequence (ITS) region located on the nuclear genome were utilised for DNA based studies. A region of the trnH-psbA gene was amplified using primer pairs trnH (5′-CCGCGATTTGTGATTACAACTCAAT-3′) and psbA (5′-GTATATCTGTGACGTTATGCTC-3′) (Sang et al., 1997). The matK region was amplified using primer pairs 1R (5′-ACCCAGCTCCATGGAAATCTGTTC-3′) and 3F (5′-CGTACAGTACTTTGTGTTTACGAG-3′) (CBOL Plant Working Group, 2009). The rbcL region was amplified using primer pairs rbcL-N (5′-ATGGTACCAAAAAAGARACKAAAG-3′) and rbcL-1R (5′-GGTTGGCCCTAAGTCCCTCC-3′) (Treutlein et al., 2003). The ITS region was amplified using primer ITS 1 (5′-TCCGCTAGTGAACCTGCGG-3′) and Chromo 5.8R (5′-GATTCTGCAATTCAC-3′) (Ramdhani et al., 2011). Each PCR reaction consisted of 4 U of DreamTaq™ DNA Polymerase, 100 mM KCl, 20 mM Tris–HCl pH 8.8, 20 mM MgCl2, 80 mM dNTP, 0.4 mM primer and approximately 20 ng of template DNA. Thirty amplification cycles were performed on a Biorad minicycler. An initial denaturation step was performed at 95°C for 5 min followed by a denaturation step at 94°C for 1 min, annealing at 55°C for 1 min and primer extension at 72°C for 1 min. The final primer elongation was done for 10 min at 72°C. PCR products were separated on a 1.5% (w/v) ethidium bromide-stained agarose gel and the bands were visualised under UV illumination.

2.5. DNA sequencing

PCR amplicons were purified using Fermantas PCR purification kits following the manufacturers’ instructions. DNA sequencing reactions
were done using a Big Dye Terminator v. 3.1 Cycle Sequencing Kit (Applied Biosystems) following the protocol outlined by the manufacturers. Sequencing products were purified by ExoSAP method (Werle et al., 1994). All DNA sequences were determined with an ABI 3500 DNA sequencer (Applied Biosystems) at Inqaba Biotechnical Industries (Pty) Ltd., South Africa.

2.6. Phylogenetic analysis

2.6.1. Sequence editing and alignment

Forward and reverse sequences were assembled and edited using CLC Main Workbench Version 7.6 (https://www.qiagenbioinformatics.com/). All sequences were deposited in GenBank (Table 2). DNA sequences were aligned using the online version of MAFFT 5.66 (Katoh et al., 2005) using the iterative refinement method and the following settings: Needleman–Wunsch algorithm active, 2 tree rebuilding steps, 1000 iterations and the program's default values for gap opening and gap extension penalties. No further manual manipulation of the alignment was performed. Sequences from Aloe, Haworthia and Gasteria species were retrieved from the GenBank and used in the phylogenetic analysis (Table 2).

2.6.2. Phylogenetic analyses

Maximum parsimony (MP) and Bayesian inference (BI) methods were used in phylogenetic reconstruction. For each analysis, the cpDNA (trnH-psbA, matK and rbcL) and nrITS sequence matrices were treated separately first and then combined. Congruence of the cpDNA and ITS datasets was assessed using the partition homogeneity (ILD) test (Farris et al., 1994) implemented in PAUP* v.4.0b10 (Swoford, 2002) and using 1000 replicates. Maximum parsimony searches were performed with PAUP*. Gaps were treated as missing characters and cladograms were generated using heuristic searches based on 1000 random sequence additions, TBR branch swapping and MULPARS effective. Bootstrap analyses were done using the same settings with a 100 replicates. Bayesian inference was performed using MrBayes v.3.1.1 (Huelsenbeck and Ronquist, 2001). The nucleotide substitution model that best fit each sequence matrix was determined with MrModeltest v.2.2. (Nylander, 2004) with model selection based on the Akaike information criterion (AIC). The MCMC run in MrBayes was set to $5 \times 10^6$ generations with tree sampling every 100th generations. The log-likelihood scores for the sampled trees were used to determine the number of generations that could be excluded before stationary has been reached (i.e. the “burn-in” value). Effective sampling size (ESS) values were assessed in Tracer version v1.5 (http://tree.bio.ed.ac.uk/software/tracer/) as a further means to determine convergence. The trees generated were viewed in FigTree v.1.4.0 (http://tree.bio.ed.ac.uk/software/figtree/) to obtain tree topologies and the posterior probability values for their nodes.

### Table 2

| Species          | Accession Number (NCBI) | matK        | psbA-trnH    | rbcL    | ITS          |
|------------------|-------------------------|-------------|--------------|---------|--------------|
| A. arborescens   | KX270415                 | KX270423    | KX270419     | KX270427 |
| A. vera          | KX270415                 | KX270424    | KX270420     | KX270428 |
| H. cymbiformis   | KX270415                 | KX270425    | KX270421     | KX270429 |
| H. fasciata     | KX270415                 | KX270426    | KX270422     | KX270430 |
| H. angustifolia  | KX270415                 | KX270423    | KX270419     | KX270427 |
| G. gloomera     | KX270415                 | KX270424    | KX270420     | KX270428 |
| Aloe             | KX270415                 | KX270425    | KX270421     | KX270429 |

### Table 3

DPPH radical scavenging activity of Mascarene Aloe species.

| Samples | DPPH radical scavenging activity IC<sub>50</sub> value (mg/ml)* |
|---------|---------------------------------------------------------------|
| Standard (Quercetin) | 0.110 ± 0.009 |
| A. purpurea (Mauritius) | 0.441 ± 0.034 |
| A. tormentari | 0.413 ± 0.014 |
| A. macra | 0.389 ± 0.007 |
| A. purpurea (Reunion) | 0.334 ± 0.009 |
| A. vera | 0.469 ± 0.010 |

* Mean ± SD of three determinations.

2.7. DPPH radical scavenging assay

Radical scavenging activity of plant extracts against stable 2, 2 diphenyl 1 picryl hydrazyl hydrate (DPPH) was determined using a modified method of Wong et al. (2006). The control was composed of methanol and DPPH solution while blanks contained methanol instead of DPPH solution. Quercetin was used as the standard.

Free radical scavenging activity was expressed as inhibition percentage and was calculated using the following formula,

\[ \% \text{Inhibition} = \frac{[1-(A_0 - A_1)/A_0]}{100} \]

where:

- $A_0$ absorption of control
- $A_1$ absorption of test samples

3. Results

3.1. NMR fingerprinting

The chemical profiling of indigenous Mascarene Aloe was performed by $^1$H NMR. The $^1$H NMR data were subjected to PCA aiming to highlight the metabolic differences among the species. PCA analysis clearly distinguished Aloe vera from the Mascarene Aloes bases on their sugar profile (3.5–4.2 ppm) (Fig. 1). Fig. 2 indicates 3 principal clusters: A. macra, A. vera and A. purpurea/A. tormentari group.

3.2. PCR amplification of the chloroplast and ITS 1 regions

The chloroplast DNA (cpDNA) and ITS 1 regions yielded single amplicons for all taxa. The PCR amplification of the cpDNA yielded amplicons of sizes 1000 bp for matK, 700 bp for psbA-trnH and 1000 bp for rbcL. Smaller amplicons of 500 bp were obtained for the ITS 1 region.

3.3. Phylogenetic reconstruction

Statistics from the MP analysis of individual chloroplast genes, ITS1, combined chloroplast only genes (matK, psbA-trnH and rbcL), and combined chloroplast and ITS datasets are shown in Table 4. Combining the chloroplast genes and ITS yielded a $P = 0.0100$ using the PHT test in PAUP, indicating that some of the genomic regions are phylogenetically incongruent. Through a process of elimination, the psbA-trnH gene was discovered causing incongruence in the combined data set. A PHT test of the combined matK, rbcL and ITS1 sequence matrices yielded a $P = 0.2300$, indicating congruence and a matrix containing these genomic regions were subsequently used in phylogenetic analyses.

 Parsimony analysis of the combined dataset yielded 68 most parsimonious trees with tree length = 68 steps, consistency index (CI) = 0.8971 and retention index (RI) = 0.9300 (Fig. 4). Phylogenetic trees generated from parsimony and Bayesian analysis clustered the Aloe species in a monophyletic group (bootstrap = 100%, posterior probability (PP) = 1) (Fig. 3). On this tree the Mascarene Aloes formed
a monophyletic group with strong nodal support (bootstrap = 92%, PP = 1). *A. tormentorii* grouped with *A. macra* in a monophyletic clade with 75% bootstrap support and PP = 1. *A. purpurea* from Reunion Island grouped sister with this group with strong support from the Bayesian analysis (PP = 0.98), but was not supported with bootstrap (54%). Contrary to what was expected, *A. purpurea* from Mauritius did not form a monophyletic group with *A. purpurea* from Reunion Island, but branched off separately from the *A. macra*, *A. tormentorii* and *A. purpurea* (from Reunion) clade.

Phylogenetic analysis of the ITS region was conducted to determine the phylogenetic signal of this region in comparison to the chloroplast genes. The MP analysis of the ITS I sequences yielded 39 parsimonious trees with tree length = 40 steps, CI = 0.900 and RI = 0.9310. (Fig. 3). The topology of the trees generated from parsimony and Bayesian analyses reflected the trees obtained from the combined data matrix (Fig. 4).

### 3.4. Antioxidant assay

Methanolic extracts of the Mascarene *Aloe* species studied showed superior free radical scavenging activities (0.334 ± 0.009 to 0.441 ± 0.034) (p < 0.05) than *A. vera* (0.469 ± 0.010) (Table 3). However, the superior radical scavenging was weaker than the positive control Quercetin with an IC₅₀ of 0.110 ± 0.009. Results from the assay showed that *A. purpurea* (Reunion) exhibited highest radical scavenging potentiality, followed by *A. macra*, *A. tormentorii*, *A. purpurea* (Mauritius) and *A. vera* (reference standard) (Table 3).

### 4. Discussion

In this study we highlight the uniqueness of the Mascarene Aloes and their phylogenetic relationships with respect to *Aloe vera* and the *Haworthia* species from the Asphodolaceae family. A previous study (Ranghoo-Sanmukhiya et al., 2010) reported the use of RAPD markers to make species delineations and have validated the use of the targeted Aloes in the folk medicine of Mascarene Islands. In this study, we used DNA sequences from chloroplast genes and the ITS region of the nuclear genome, 1H-NMR fingerprinting and antioxidant assays to characterise the Mascarene Aloes. Results from this study highlighted the distinctiveness of Mascarene Aloes with respect to *Aloe vera*.

Maximum parsimony analysis of the chloroplast genes (tree not shown) showed that gens from this genome and used in this study
Fig. 2. Score plot of PCA results obtained from all 1H NMR data showing PC1 and PC2. AV: Aloe vera; AM: Aloe macra, AT: Aloe tormentorii, AP1: Aloe purpurea Mauritius, AP2: Aloe purpurea Reunion.

Fig. 3. One of the 39 most parsimonious tree obtained from the maximum parsimony (MP) analyses of the ITS 1 dataset. *Gasteria glomerata* was used as outgroup. Numbers above branches indicate MP bootstrap values, while numbers below the branches indicated the posterior probability values obtained from a Bayesian analysis. Scale bar: Number of steps.
are less informative compared to the ITS I region to elucidate the close relationships which exist among the Mascarene Aloes. Parsimony analysis of the ITS and combined datasets separately revealed a polytomy at the node that is shared by A. purpurea from Reunion and Mauritius, respectively, and A. macra and A. tormentorii formed a monophyletic group. The non-informative characteristic of the chloroplast DNA rbcl and matK has been previously reported (Gielly and Taberlet, 1994; Muller et al., 2006). However, the variable nature of the psbA-trnH intergenic spacer has also been reported previously (Ramdhani et al., 2011), and therefore it was expected that this region would be more variable than the other genes from the chloroplast genome. A combined cpDNA (psbA-trnH, matK and rbcl) and ITS1 phylogeny was thus used to obtain a finer resolution of the taxonomy of the Mascarene Aloes.

In this study the combined dataset including the chloroplast genes and ITS1 yielded a phylogeny that separated most of the Mascarene Aloe species with strong support. The trees generated in this study clustered the Mascarene Aloes in a strongly supported monophyletic group. Within this group, A. tormentorii formed a monophyletic group with A. macra and A. purpurea from Reunion Island being a sister species to this group as supported by the Bayesian posterior probability for the node. Interestingly, A. purpurea from Reunion Island was placed paraphyletic to A. purpurea from Mauritius and basal to A. macra and A. tormentorii. A. purpurea from Reunion Island is morphologically distinct from the A. purpurea of Mauritius (Ranghoo-Sanmukhiya et al., 2010) and we postulate that this Aloe could possibly be a hybrid of A. macra and A. tormentorii. A. purpurea from Reunion Island is morphologically distinct from the A. purpurea of Mauritius (Ranghoo-Sanmukhiya et al., 2010) and we postulate that this Aloe could possibly be a hybrid of A. macra and A. tormentorii. A. purpurea from Reunion Island has been proposed in a previous study (Ranghoo-Sanmukhiya et al., 2010). The possibility of A. purpurea from Reunion being a hybrid of A. macra and A. tormentorii has been proposed in a previous study (Ranghoo-Sanmukhiya et al., 2010).

The NMR profile and score plot of PCA results, based on expression of genes clearly differentiated the three indigenous Mascarene species from A. vera. The discrimination, in the NMR analysis, between A. macra and other Aloe species, was correlated with sugar signals (3.5–4.2 ppm), indicating a higher sugar content in A. macra. PCA is an unsupervised clustering method which necessitates no knowledge of the data set and aims at reducing the dimensionality of multivariate data but preserves most of the variance within it (Eriksson et al., 2001). In the present study, the two origins of A. purpurea were clearly discriminated in their NMR profile based on their sugar content. The presence of saccharides such as arabinose, galactose, glucose, mannose and xylose were previously reported to occur in Aloe species (Davis, 1997). From PCA analysis based on sugar content, A. purpurea and A. tormentorii from Mauritius seem to have produced the same metabolites probably due to their exposure to similar environmental conditions.

The trends observed with the NMR fingerprinting were also reflected in the antioxidant capacity of the studied Aloes. More similar lower IC50 values were observed between A. purpurea and A. macra from Reunion than between Mauritian Aloes and A. vera. Results from this study showed that A. purpurea from Mauritius had less important antioxidant activity than Reunion Island Aloes, the IC50 value was still lower than that of A. vera.

The correlation between the polyphenolic profile and antioxidant capacity of natural products is well established (Mihai et al., 2011). The present findings agree with an earlier study (Ranghoo-Sanmukhiya et al., 2010) in which the HPLC and TLC profiles of A. vera were completely different from that of the Mascarene Aloes, especially with respect to the flavonoid content. The increasing interest in the search for natural antioxidants has focused on those containing flavonoids and phenolic acids reputed to fight free radical damage (Gulcin et al., 2010). The antioxidant activity of phenolics is mainly due to their redox properties, thereby acting as reducing agents with free radicals scavenging abilities (Mihai et al., 2011). The 2, 2 diphenyl 2 picryl hydrazyl hydrate (DPPH) is a stable and commercially available source of organic nitrogen radicals and is widely used to assess the radical scavenging activity of antioxidant compounds. In a previous study (Ko et al., 1998; Wang et al., 2012), the renowned antioxidant, isoorientin, was only detected in the putative hybrid A. purpurea (IC50 0.334 ± 0.005) and in A. macra (IC 50: 0.389 ± 0.007) from Reunion Island and not in the other Mascarene Aloes (Ranghoo-Sanmukhiya et al., 2010).

Fig. 4. One of the 68 most parsimonious trees obtained from the maximum parsimony (MP) analyses combined matK, rbcl and ITS 1 matrix. Gasteria glomerata was used as outgroup to root the tree. Numbers above branches are MP bootstrap values, while numbers below the branches are posterior probability values obtained from a Bayesian analysis. Scale bar: Number of steps.
et al., 2010). This could explain the higher antioxidant capacity of the Aloes of Reunion Island as compared to their other Mascarene counterparts.

5. Conclusion

The multidisciplinary approach followed in this study by integrating 1H NMR/PCA, antioxidant and phylogenetic analysis was complementary and has proven a very promising tool for discriminating between Aloe species of common origin. Simple PCA used for the reduction of the NMR data set obtained from the metabolites corroborated with the antioxidant assay. The superior antioxidant capacity of the Mascarene Aloes over A. vera is pioneering. Furthermore, phylogenetic studies have confirmed the uniqueness of the Mascarene Aloes relative to Aloe vera. Results, based on antioxidant profile from this study validated their medicinal virtues. The Mascarene Aloes warrant further research as they represent an untapped source of natural products with potential for pharmaceutical application.

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