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Combination of LC-MS/MS and Molecular Networking in Investigating the Phytochemicals in *Murraya koenigii*

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Murraya koenigii has been used in traditional Indian Ayurvedic medicine and in culinary as flavouring agents. The metabolites in the leaves of *M. koenigii* have been studied extensively for its pharmacological properties, revealing a diverse range of its therapeutic effects. This study aimed to investigate the metabolites in the leaf, bark, and root of *M. koenigii* using a combination of Liquid Chromatography-Tandem Mass Chromatography (LC-MS/MS) and molecular networking as a dereplication strategy for the detection of potential new compounds. The comprehensive metabolite profiling of the *M. koenigii* dichloromethane extracts was carried out by generating a molecular network from LC-MS/MS data. A total of 47 known compounds and 11 unknown compounds were putatively identified based on their molecular structure obtained from the analysis of the MN. There were six clusters in the MN by which five clusters were composed of alkaloids and one cluster was consisted of terpenoids. The present study suggested that three alkaloid compounds putatively identified for the first time in the bark and root of *M. koenigii* might appear to be potential bioactive compounds deserving of further investigation. The two compounds, (1-(4-ethoxybutyl)-2-methyl-1H-indol-3-yl) (p-tolyl) methanone (peak **2**) and 1,4-bis(3-phenylpropyl)-1,2-dihydropyridine (peak **4**), were found exclusively in the bark, whereas 2-methyl-4,6-bis(4-(pyridin-4-yl) phenyl)-1,3,5-triazin (peak **3**) was found abundantly in the root. These compounds could be potential candidates for future research, including targeted compound isolation, followed by structural elucidation and bioassays, to be further explored as a potential lead compound in drug discovery.

Keywords: Molecular networking; dereplication; curry leaf; bark and root; dichloromethane extract

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Murraya koenigii, commonly known as curry leaf plant, is a tropical to subtropical species in the family Rutaceae. It is native to South Asia, with its primary distribution in countries such as India, Sri Lanka, Andaman Islands, Thailand, Nepal, Vietnam, Malaysia, South Africa, and Reunion Island [1]. It is rich in various phytochemicals that contribute to its distinctive flavour, aroma, and potential health benefits. These phytochemicals imbue the barks, leaves and roots with distinctive properties, making them highly sought-after in both culinary and medicinal applications [2]. Curry leaves have a long history of being used as a flavouring agent in South Asian and Southeast Asian recipes. It is commonly used as a seasoning agent in Indian curries, soups, salads, chutneys, and other dishes, to promote appetite and digestion due to the presence of volatile oil [3]. However, their benefits extend beyond the culinary realm [1].

Over the centuries, *M. koenigii* has been used in many forms and in traditional Indian Ayurvedic medicine, known as "krishnanimba" in the treatment and prevention of diseases. The bioactivity of curry leaves is linked to the presence of various phyto-chemicals, such as alkaloids, essential oils, phenolics, terpenoids, tocopherol, β-carotene, and lutein [2]. In addition, curry leaves contain essential minerals, proteins, and fats, contributing to their overall nutritional profile [4]. *M. koenigii* has a variety of therapeutic activities. It is used to alleviate symptoms of dysentery, diarrhoea, body aches, fresh cuts, kidney pains, vomiting, reducing inflammation and itching. Curry leaf extracts have been used to address calcium and vitamin deficiencies, as well as anaemia. It has been extensively studied for its pharmacological properties, revealing a diverse range of therapeutic effects. The extract demonstrates notable antioxidant, anti-inflammatory, antifungal, anti-protozoal, anticancer, immunomodulatory, skin radiation protection, nephroprotective, antipyretic, anthelmintic, anti-ulcer, anti-osteoporotic, cardioprotective, inotropic, mosquitocidal, and larvicidal activities [5, 6].

Despite the benefits of *M. koenigii*, there are limited studies on the leaf, bark, and root of this plant

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by using molecular networking (MN) as a dereplication strategy. Since plant metabolomes are complex and diverse, molecular networking has emerged as a remarkable tool for handling complex mass spectrometry (MS) data effectively [7]. It was introduced for the prioritization of large collections of bioactive natural extracts and the annotation of bioactive fractions [8]. It facilitates the grouping of molecules with similar structures by comparing the similarity of their MS/MS fragments. The compounds that share similar MS/MS fragmentation patterns or molecular classes tend to cluster together in MN. This advantageous feature significantly improves the identification of unidentified nodes, particularly when their spectra or the spectra of neighbouring nodes are known and annotated after the comparison with public or internal library databases. Therefore, the combination of LC-MS/MS and molecular networking techniques offers a substantial enhancement in efficiency while drastically reducing the data processing time by avoiding the isolation of known compounds [9].

In recent years, several software tools have played a vital role in advancing the field of molecular networking and natural product research. Among these tools, MZmine 3, SIRIUS, GNPS, and Cytoscape have emerged as prominent platforms for data processing, compound annotation, molecular networking, and network visualization, enabling researchers to explore and understand complex chemical landscapes with greater efficiency and accuracy. In this study, the capabilities and contributions of these software tools in molecular networking and natural product research will be explored. Therefore, the objective of this study was to investigate the metabolites in the leaf, bark, and root of *M. koenigii* using a combination of LC-MS/MS and molecular networking as a dereplication strategy for the detection of potential new compounds.

EXPERIMENTAL

Materials and Methods

Preparation of Crude Extracts

The *M. koenigii* specimens were obtained from Sibu, Sarawak, Malaysia by botanical researchers of Universiti Pendidikan Sultan Idris (UPSI). Authentication of the plant specimens was conducted by Mr. Teo Leong Eng, the botanist from Herbarium Group, Univerisiti Malaya (UM), Kuala Lumpur, Malaysia in July 2022. The assigned voucher number for the specimens *M. koenigii* was TM1008. The voucher specimens were kept in herbarium of the Department of Chemistry, Universiti Pendidikan Sultan Idris (UPSI), located in Perak, Malaysia. The dried dichloromethane crude extract of *M. koenigii* leaf, bark and root were obtained via cold maceration method, utilizing 80% dichloromethane (Supelco, Germany). Firstly, all the samples were weighed, washed with running tap water, and allowed to dry in oven at 40 °C. Then, the dried samples were ground into powder using an electric blender. A total of 600 g of finely ground leaves, 2400 g of finely ground barks and 780 g of finely ground root samples were extracted with 2L of 80% analytical grade dichloromethane for six days, respectively. Half of the dichloromethane crude extract was decanted and replaced with the same amount of freshly prepared 80% dichloromethane every two days. The dichloromethane leaf, bark and root crude extracts collected were filtered through filter paper and concentrated *in vacuo* at 40 °C using a rotary evaporator (Heidolph, Hei-VAP Value Digital).

Liquid Chromatography-Mass Spectrometry (LC-MS) Analysis

All the samples, including the dichloromethane leaf (DL), bark (DB) and root (DR) extracts were prepared by dissolving 1 mg of each dried crude extract in 1 mL of High-Performance Liquid Chromatography (HPLC) grade methanol, respectively. All the samples were filtered through 0.22 µm nylon syringe filter before analysed by using an Agilent 1290 Infinity LC system coupled to an Agilent 6520 Accurate-Mass Q-TOF mass spectrometer with dual ESI source. The analysis employed an Agilent Eclipse XDB-C18 Narrow-bore column (150 mm × 2.1 mm, 3.5-micron, P/N 930990-902) at a column temperature of 25 °C. The flow rate was set to 0.5 mL/min, and the sample injection volume was 3.0 µL. The LC system used a gradient program with solvents A (0.1% formic acid in water) and B (0.1% formic acid in acetonitrile). The mass spectrometer was operated in positive ionization modes with MS and autoMS/MS (aMS/MS) mode by applying the following parameters: Vcap voltage of 4000 V, fragmentor voltage set at 125 V, skimmer voltage of 65 V, and OCT 1 RF Vpp of 750 V. The drying gas was maintained at a flow rate of 10 L/min and a temperature of 300°C, while the nebulizer pressure was set to 45 psig. The mass range for MS analysis was set to a minimum of 100 m/z, while for aMS/MS, it was set to a minimum of 50 m/z, both with a maximum of 3200 m/z. Reference ions at 121.0508 and 922.0097 were utilized during the analysis. The acquisition rate was 1.03 spectra/s for MS and 4 spectra/s for aMS/MS, with an acquisition time of 970.9 ms/spectrum for MS and 250 ms/spectrum for aMS/MS. For both MS and aMS/MS, the number of transients per spectrum was 9663 and 2330, respectively. Collision energy was varied at 10, 20, and 40 volts.

Feature-Based Molecular Networking (FBMN)

The raw MS and MS/MS data file (.d file) for blank MeOH, DL, DB and DR obtained from the Agilent instrument were converted to mzML format using MSConvert from ProteoWizard Tools (https://proteowizard. sourceforge.io/). In this study, Mzmine 3 (Version: 3.1.0 beta) (http://mzmine.github.io/) was employed for spectral feature detection and annotation by database in spectral library search (MassBank of North America library, Fiehn/Vaniya natural product library and GNPS library). Mass detection was performed with noise level at 1.0E3 for MS1 and 0.0E0 for MS2. The chromatogram was built with retention time within 0.50-24.00 minutes, group intensity threshold at 5.0E5, and minimum highest intensity at 7.0E5. Chromatographic resolver was conducted by employing a baseline cutoff algorithm with minimum feature height at 500000, peak duration range at 0.010–1.00 minutes, retention time wavelet range within 0.00-0.10, S/N threshold and coefficient/area threshold were set at 10, respectively. The chromatograms were deisotoped by an isotope filter at an m/z tolerance of 0.0010 m/z or 5.000 ppm and retention time tolerance at 0.030 minutes. Alignment was performed using a join aligner module with m/z tolerance at 0.0010 m/z or 5.000 ppm, retention time tolerance at 0.100 minutes, and the weight for m/z and retention time at 3 and 1, respectively. The peak list was gap-filled with a peak finder module (intensity tolerance 20.0%, m/z tolerance of 0.0010 m/z or 5.000 ppm, and the retention time tolerance at 0.100 minutes).

The spectral information (MGF file) was generated for further data processing. The processed data file was subjected to compound annotation using SIRIUS software (Version: 5.7.2) (https://bio.informatik.uni-jena.de/software/sirius/). The identification and annotation of compounds from mass spectrometry data were conducted using Orbitrap at MS2 mass accuracy (10 ppm), candidates stored (10), minimum candidates per ion stored (1), use heuristic above m/z and use heuristic only above m/z (300 and 650). All the structure databases listed were selected in the compound annotation process (Bio Database, CHEBI, COCONUT, GNPS, HMDB, KNApSAcK, PubChem, PubMed etc.). The annotation results were evaluated by COSMIC and CANOPUS predicted compound class based on molecular fingerprint generated by the CSI Finger ID.

Further analysis and interpretation were carried out using GNPS platform (https://gnps.ucsd.edu/ProteoSAFe/static/gnps-splash.jsp; accessed in 6 July 2023). The precursor and fragment ion mass tolerance were set to 0.02 Da, respectively. MNs networks were generated using six minimum matched peaks and a minimum pairs cosine score of 0.7. The mass spectrometry spectral data within the network were subsequently searched for in the spectral library of GNPS. The molecular network was visualized using Cytoscape 3.10 (https://cytoscape.org/). All the compound 2D-structures were visualized in chemViz2 (https://apps.cytoscape.org/apps/chemviz2) and the identity for each compound structures were revealed with ChemDraw Professional 16.

RESULTS AND DISCUSSION

The metabolite profiling of the *M. koenigii* leaf, bark and root extracts was carried out using LC-MS/MS. A total of 22, 24, and 22 chromatographic peaks were annotated in the leaf, bark, and root extracts, respectively. The LC-MS analysis was conducted in positive mode and the compounds were identified based on their full MS and MS/MS spectra obtained in the positive ion mode. The LC-MS/MS revealed the diverse alkaloids and lipid compounds found in the leaf, bark, and root extracts of *M. koenigii*.

Molecular networking served as a tool in data mining through the large data set via the clustering of the MS/MS spectra based on chemical similarities. The molecular network (MN) of the leaf, bark and root extract was generated from the LC-MS/MS analysis data to analyse the metabolite content of *M. koenigii*. The MN revealed the abundance of alkaloid compounds and lipids in the extracts. Figure 1 shows the MN generated from the *M. koenigii* extracts with six different clusters, whereby the compounds in each cluster shared similar molecular formula, phytochemical classes, or fragmentation patterns. The putative identification of the compounds was carried out by matching the data to the different mass spectroscopic databases, such as PubChem (https://pubchem.ncbi.nlm.nih.gov/), HMDB (https://hmdb.ca/), and KNapSAcK (http://www.knapsackfamily.com/KNApSAcK/). Table 1 showed a total of 47 known compounds and 11 unknown compounds that were putatively identified based on their molecular structure obtained from the analysis of the MN. The structure of the putatively identified unknown compounds were shown in the MN. Clusters 1 to 5 were composed of alkaloid compounds, while cluster 6 was consisted of two terpenoid compounds. Out of the 47 known compounds, only four were previously isolated from *M. koenigii*, namely mahanimbine, mahanine, girinimbine and caryophyllene oxide [10].

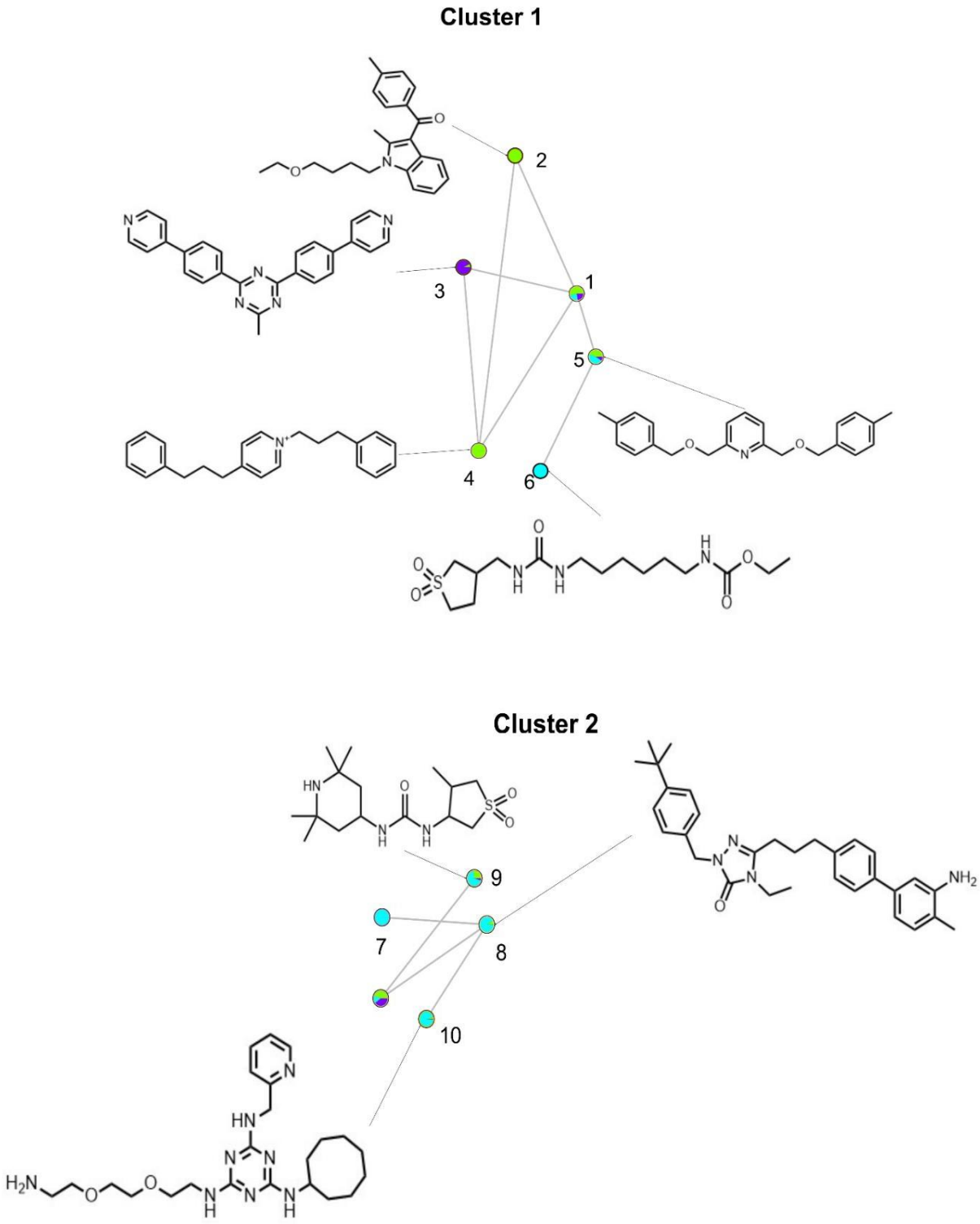


Figure 1. Annotation of the molecular network of the metabolites derived from *Murraya koenigii* leaf, bark and root extracts. The chemical structures are putatively identified structure of the unknown compounds.

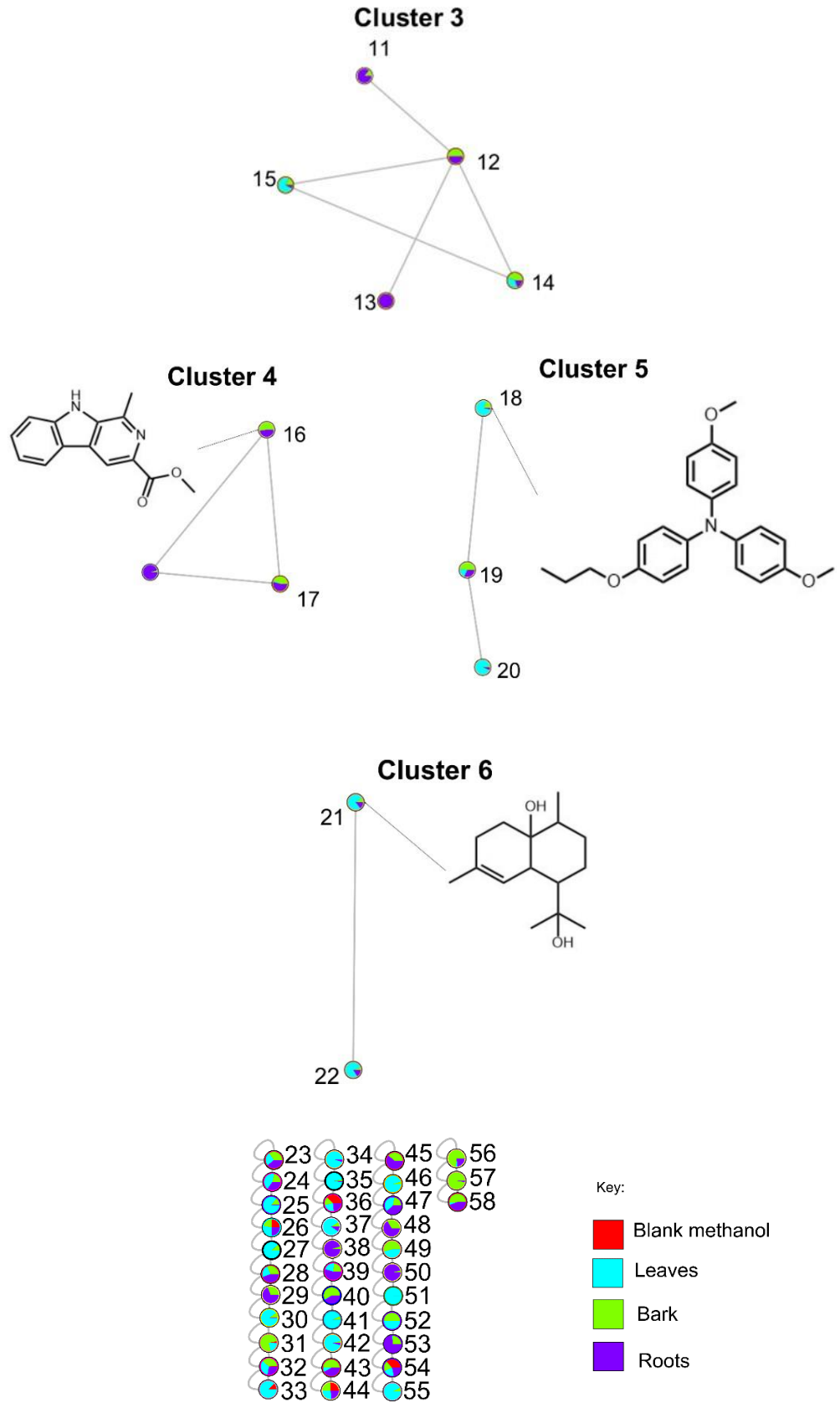


Figure 1. Continued.

Table 1. Annotated compounds based on LC-MS/MS and MN in the leaf, bark and root extracts of *Murraya koenigii*.

Peak number	Putative compounds	Molecular formula	Composition in plant (%)	Retention time (Rt)	Adducts	Calcd. $[M + H]^+$ (m/z)	Exp. $[M + H]^+$ (m/z)	Mass error (m/z)	MS/MS (m/z)	References
				Cluster 1						
1	2-(3-benzyl-2-methyl-1-propylindol-5-yl) oxy-2-methylpropanoic acid	$C_{23}H_{27}NO_3$	Leaf (24%) Bark (54%) Root (22%)	14.69	$[M + H]^+$	365.4653	366.2064	0.7411	210.0913	[11]
2*	(1-(4-ethoxybutyl)-2-methyl-1H-indol-3-yl) (p-tolyl) methanone	$C_{23}H_{27}NO_2$	Bark (100%)	17.98	$[M + H]^+$	349.4660	350.2110	0.7450	211.0932 210.0891 182.0942	-
3*	2-methyl-4,6-bis(4-(pyridin-4-yl) phenyl)-1,3,5-triazine	$C_{26}H_{19}N_5$	Bark (8%) Root(92%)	18.79	$[M +H_2O+H]^+$	401.4626	420.1849	18.7223	211.0984 210.0913 167.0716	-
4*	1,4-bis(3-phenylpropyl)-1,2-dihydropyridine	$C_{23}H_{25}N$	Bark (100%)	18.09	$[M +H_2O+H]^+$	315.4513	334.2174	18.7661	210.0913 182.0936	-
5*	2,6-bis(((4-methylbenzyl) oxy) methyl) pyridine	$C_{23}H_{25}NO_2$	Leaf (46%) Bark (44%) Root (10%)	16.51	$[M + H]^+$	347.4501	348.1967	0.7466	210.0913	-
6*	ethyl (6-(3-((1,1-dioxidotetrahydrothiophen-3-yl) methyl) ureido) hexyl) carbamate	$C_{15}H_{29}N_3O_5S$	Leaf (100%)	14.34	$[M + H]^+$	363.4729	364.1907	0.7178	226.0863 210.0913	-
				Cluster 2						
7	2-(3,5,7-tripyridin-2-ylcyclooctyl) pyridine	$C_{28}H_{28}N_4$	Leaf (100%)	21.89	$[M +C_3H_8O+H]^+$	420.5487	481.2991	60.7504	264.1006	[11]
8*	5-(3-(3'-amino-4'-methyl-[1,1'-biphenyl]-4-yl) propyl)-2-(4-(tert-butyl) benzyl)-4-ethyl-2,4-dihydro-3H-1,2,4-triazol-3-one	$C_{31}H_{38}N_4O$	Leaf (92%) Bark (8%)	21.48	$[M + H]^+$	482.6596	483.3135	0.6539	400.2291	-
9*	1-(4-methyl-1,1-dioxidotetrahydrothiophen-3-yl)-3-(2,2,6,6-tetramethylpiperidin-4-yl) urea	$C_{15}H_{29}N_3O_3S$	Leaf (68%) Bark (28%) Root(5%)	19.43	$[M + H]^+$	331.4741	332.2014	0.7273	250.1226 249.1148	-
10*	N2-(2-(2-(2-aminoethoxy) ethoxy) ethyl)-N4-cyclooctyl-N6-(pyridin-2-ylmethyl)-1,3,5-triazine-2,4,6-triamine	$C_{23}H_{38}N_8O_2$	Leaf (95%) Bark (4%) Root(1%)	22.77	$[M +Na]^+$	258.6002	481.2994	222.6992	398.2165 264.1010	-
				Cluster 3						
11	5,7-dihydro-6H-dibenzo[d,f][1,3]diazepin-6-one	$C_{13}H_{10}N_2O$	Bark (14%) Root (86%)	10.42	[M + H] +	210.2313	211.0865	-0.8552	168.0808 167.0725	[11]
12	4-Methyl-acridone	$C_{14}H_{11}NO$	Bark (50%) Root(50%)	11.83	$[M + H]^+$	209.2432	210.0915	0.8483	167.0730 166.0615	[11,12]
13	Acridone	$C_{13}H_9NO$	Root (100%)	12.82	$[M + H]^+$	195.2167	196.0754	3.8587	168.0808 167.0722	[11]
14	Benzacridine	$C_{17}H_{11}N$	Leaf (37%) Bark (43%) Root (20%)	16.33	$[M +H_2O+H]^+$	229.2759	248.1071	-24.8312	204.0808 205.0865	[11]

15	Benza(b)cridine	$C_{17}H_{11}N$	Leaf (75%) Bark (18%) Root (8%)	16.72	$[M+H_2O+H]^+$	229.2759	248.1075	-18.8316	204.0808 205.0865	[11]
				Cluster 4						
16*	methyl 1-methyl-9H-pyrido[3,4-b] indole-3-carboxylate	$C_{14}H_{12}N_2O_2$	Bark (52%) Root (48%)	10.95	[M + H] +	240.2573	241.0972	0.8399	198.0921 183.0679, 167.0730, 154.0651	-
17	2-methoxy-10H-acridin-9-one	$C_{11}H_{17}NO_2$	Leaf (3%) Bark (42%) Root (54%)	13.41	$[M+H]^+$	225.2464	226.0865	0.8401	198.0900 183.0681 167.0651, 154.0651	[11]
				Cluster 5						
18*	4-methoxy-N-(4-methoxyphenyl)-N-(4-propoxyphenyl) aniline	$C_{23}H_{25}NO_3$	Leaf (79%) Bark (17%) Root (3%)	16.78	$[M+H]^+$	363.4495	364.1919	0.7424	248.1070 226.0863 123.1168	-
19	Mahanimbine	$C_{23}H_{25}NO$	Leaf (21%) Bark (48%) Root (32%)	19.73	$[M+H]^+$	331.4507	332.2013	0.7506	250.1232 249.1104 248.1059	[13]
20	Mahanine	$C_{23}H_{25}NO_2$	Leaf (90%) Bark (4%) Root (6%)	17.80	$[M+H]^+$	347.4501	348.1965	0.7464	265.1058 264.1019	[14]
				Cluster 6						
21*	1-(2-hydroxypropan-2-yl)-4,7-dimethyl-1,3,4,5,6,8a-hexahydronaphthalen-4a(2H)-ol	$C_{15}H_{26}O_2$	Leaf (75%) Bark (10%) Root (14%)	17.39	$[M-H_2O+H]^+$	238.3657	221.1900	-17.1757	203.1794	-
22	Bisabolol oxide A	$C_{15}H_{26}O_2$	Leaf (79%) Bark (5%) Root (16%)	17.21	$[M-H_2O+H]^+$	238.3657	221.1901	-17.1757	203.1794	[11]
				Single nodes						
23	Adogen 42	$C_{18}H_{37}NO$	Leaf (26%) Bark (35%) Root (39%)	21.31	$[M+H]^+$	283.4925	284.2949	0.8024	57.0699	[11]
24	9-hydroxystearic acid	$C_{18}H_{36}O_3$	Leaf (44%) Bark (21%) Root (35%)	12.23	$[M+H_3N+H]^+$	300.4766	318.3009	17.9143	102.0913, 88.0757	[11]
25	2-methylidenedecanedioic acid	$C_{11}H_{18}O_4$	Leaf (85%) Bark (14%) Root (1%)	9.31	$[M-H_2O+H]^+$	214.2582	197.1171	-17.1411	133.1012 107.0856	[11]
26	1,3-dihydro-2-benzofuran-1,3-dione	$C_8H_4O_3$	Leaf (29%) Bark (22%) Root (24%) Blank (25%)	16.98	$[M+H]^+$	148.1156	149.0235	0.9079	121.0284 65.0386	[11]
27	Cealysin	$C_9H_{18}N_6O_6$	Leaf (88%) Bark (12%)	15.92	$[M+H_3N+H]^+$	306.2758	324.1598	17.8840	278.1176 263.0941, 210.0913	[11]

28	Oleamide	$C_{18}H_{35}NO$	Leaf (24%) Bark (32%) Root (44%)	19.61	$[M + H]^+$	281.4766	282.2794	0.8028	265.2526 247.2420	[11]
29	6-(9H-carbazol-2-yloxy) hexanoic acid	$C_{18}H_{19}NO_3$	Bark (31%) Root (69%)	14.34	$[M -H_2O+H]^+$	297.3484	280.1332	17.2152	210.0913	[11]
30	(2S)-2-cyclohexylpent-4-ynoic acid	$C_{11}H_{16}O_2$	Leaf (89%) Bark (10%) Root (1%)	12.41	$[M + H]^+$	180.2435	181.1219	0.8784	107.0855 93.0699	[11]
31	Sandin EU	$C_{21}H_{42}O_4$	Leaf (21%) Bark (76%) Root (1%) Blank (3%)	21.02	$[M +Na]^+$	358.5558	381.2994	22.7436	-	[11]
32	Propanon	C_3H_6O	Leaf (33%) Bark (38%) Root (28%) Blank (1%)	1.99	$[M +C_2H_3N+H]^+$	58.0791	100.0758	41.9967	-	[11]
33	4,6-dimorpholin-4-yl-1,3,5-triazin-2-amine	$C_{11}H_{18}N_6O_2$	Leaf (88%) Blank (12%)	17.91	$[M +C_2H_3N+H]^+$	266.2996	308.1857	41.8861	186.0604 178.0499 150.0550	[11]
34	1,4,4,8-tetramethyl-12-oxatricyclo [6.3.1.02,5] dodecan-9-ol	$C_{15}H_{26}O_2$	Leaf (95%) Root (5%)	12.23	$[M -H_2O+H]^+$	238.3657	221.1900	-17.1757	203.1866 119.0815 105.0659	[11]
35	Piperidine-2-carboxylic acid	$C_6H_{11}NO_2$	Leaf (94%) Bark (3%) Root (2%)	0.70	$[M + H]^+$	129.1570	130.0861	0.9291	84.0808	[11]
36	1,8-Octandiol	$C_8H_{18}O_2$	Leaf (23%) Bark (16%) Root (23%) Blank (37%)	14.14	$[M-H_4O_2+H]^+$	146.2273	111.1165	35.1108	69.0699	[11]
37	13-Kodda	$C_{18}H_{30}O_3$	Leaf (81%) Bark (9%) Root (10%)	15.22	$[M -H_2O+H]^+$	294.4290	277.2165	-17.2125	93.0699 79.0542	[11]
38	5H-Benzo[b]carbazole,6-methyl-	$C_{17}H_{13}N$	Bark (5%) Root (95%)	16.51	$[M+CH_4O+H]^+$	231.2918	264.1384	32.8466	234.0913 194.0964 180.0808	[11]
39	7-Octadecyn-1-ol	$C_{18}H_{34}O$	Leaf (23%) Bark (23%) Root (54%)	20.08	$[M +C_2H_3N+H]^+$	266.4620	308.2952	41.8332	270.2791 72.0444	[11]
40	(8R,10S,13S)-13-methyl-1,2,5,6,7,8,9,10,11,12,14,15,16,17-tetradecahydrocyclopenta[a]phenanthren-17-ol	$C_{18}H_{28}O$	Leaf (2%) Bark (57%) Root (41%)	14.60	$[M -H_3N+H]^+$	260.4143	278.2477	17.8334	261.2213, 95.0855	[11]
41	2,4,4-triphenylpentan-2-ylbenzene	$C_{29}H_{28}$	Leaf (93%) Root (7%)	20.90	$[M +C_4H_6N_2+H]^+$	376.5326	459.2790	82.7464	308.1308	[11]
42	Caryophyllene oxide	$C_{15}H_{24}O$	Leaf (92%) Bark (4%) Root (5%)	16.16	$[M + H]^+$	220.3505	221.1896	0.8391	203.1794 91.0539	[11]

43	Amide 16	$C_{16}H_{33}NO$	Leaf (9%) Bark (49%) Root (42%)	19.38	$[M + H]^+$	255.4393	256.2640	0.8247	102.0913 88.0757 57.0699	[11]
44	Hatcol DBP	$C_{16}H_{22}O_4$	Leaf (27%) Bark (23%) Root (23%) Blank (26%)	16.98	$[M + H]^+$	278.3435	279.1599	0.8164	149.0233	[11]
45	Heptadecanamide	$C_{17}H_{35}NO$	Bark (39%) Root (61%)	19.96	$[M + H]^+$	269.4659	270.2802	0.8143	88.0757 57.0699	[11]
46	ACMC-20mngi	$C_{16}H_{11}NO$	Leaf (95%) Bark (5%)	17.80	$[M+CH_4O+H]^+$	233.2646	266.1181	32.8535	-	[11]
47	Cetylate	$C_{16}H_{32}O_2$	Leaf (40%) Bark (22%) Root (38%)	12.18	$[M -H_3N+H]^+$	256.4241	274.2747	17.8506	256.2635 106.0863 88.0757	[11]
48	Girinimbine	$C_{18}H_{17}NO$	Bark (33%) Root (67%)	17.31	$[M + H]^+$	263.3337	264.1390	0.8053	234.0913 220.0757 194.0964	[13]
49	Monopalmitin	$C_{19}H_{38}O_4$	Leaf (45%) Bark (55%)	19.50	$[M + Na]^+$	330.5026	353.2667	22.7641	85.1012 71.0855	[11]
50	1-methyl carbazole	$C_{13}H_{11}N$	Bark (4%) Root (96%)	15.16	$[M + H]^+$	181.2331	182.0961	0.8630	167.073 166.0651	[11]
51	Tectochinon	$C_{15}H_{10}O_2$	Leaf (100%)	14.57	$[M +C_2H_3N+H]^+$	222.2387	264.1022	41.8635	221.0835 220.0757	[11]
52	alpha-Linolenic acid	$C_{18}H_{30}O_2$	Leaf (51%) Bark (49%)	15.87	$[M + H]^+$	278.4296	279.2320	0.8024	95.0855 81.0699	[11]
53	13(S)-HODE methyl ester	$C_{19}H_{34}O_3$	Leaf (2%) Bark (25%) Root (73%)	18.09	$[M -H_2O+H]^+$	310.4715	293.2478	-17.2237	95.0855 81.0699	[11]
54	8-butoxyoctanoic acid	$C_{12}H_{24}O_3$	Leaf (22%) Bark (20%) Root (22%) Blank (37%)	14.46	$[M -H_2O+H]^+$	216.3172	199.1690	-17.1482	111.1168 69.0699	[11]
55	2-butoxy-8-methoxy-9- [4-(2-methoxyethylamino) butyl] purin-6-amine	$C_{17}H_{30}N_6O_3$	Leaf (93%) Bark (7%)	20.78	$[M +C_2H_6OS+H]^+$	366.4585	445.2613	78.8028	294.1111	[11]
56	3-(dimethylamino)-N-[4-methyl-3-[(3-nitrobenzoyl) amino] phenyl] benzamide	$C_{23}H_{22}N_4O_4$	Leaf (3%) Bark (74%) Root (23%)	10.60	$[M -H_2O+H]^+$	482.4428	401.1598	-81.2830	383.1503 217.0859	[11]
57	2-[[amino(nitramido)methylidene] amino]-1-nitroguanidine	$C_2H_6N_8O_4$	Bark (97%) Root (3%)	10.30	$[M +C_3H_8O+H]^+$	206.1202	267.1133	60.9931	183.0679	[11]
58	(2E,4E)-N-isobutyltetradeca-2,4-dienamide	$C_{18}H_{33}NO$	Leaf (4%) Bark (52%) Root (45%)	18.34	$[M + H]^+$	279.4607	280.2635	0.8028	263.2369 245.2264 242.2478	[11]

Note: *Unknown compounds putatively identified based on the molecular structure; 'Calcd.' Calculated, and 'Exp.' Experimental

Cluster 1 in the MN consisted of six compounds, which are heterocyclic compounds with a ring structure that contain at least one heteroatom. All the compounds in cluster 1 were characterized by the presence of a common MS/MS fragment ions of m/z 210.0913. However, due to the difference in their molecular structure, these compounds do not share a similar fragmentation pattern despite the similarity in the MS/MS fragment detected. Among the compounds, peak **1**, with RT 14.69, was identified as 2-(3-benzyl-2-methyl-1-propylindol-5-yl) oxy-2-methylpropanoic acid, a known compound with the parent ion at m/z 366.2064 $[M + H]^+$. Peaks **2**, **3** and **4**, were identified as pyridine alkaloids for the first time. Peaks **2** and **4** were found to be present exclusively in the bark, whereas peak **3** was found abundantly in the root of *M. koenigii* (Figure 2). Pyridine alkaloids are compounds with a pyridine nucleus and a pyrrolidine or piperidine unit. Pyridine-containing compounds are gaining significant importance in medicinal applications due to their diverse range of activities including antiviral, anticholinesterase, antimalarial, antimicrobial, anti-diabetic, and anticancer activities [15, 16]. The presence of the nitrogen atom in pyridine plays a crucial role in the pharmacological profile of many drugs [17]. Pharmaceutically, a pyridine-based synthesised compound enhances its biological potency, penetrability and metabolic stability, and fixes protein-binding issues. Typically, pyridine compounds are mostly originated as alkaloids [18].

The 2,6-bis(((4-methylbenzyl) oxy) methyl) pyridine (peak **5**) identified from cluster 1 is a carbazole alkaloid, present in the leaf, bark, and root extracts of the plant, whereas ethyl (6-(3-((1,1-dioxidotetrahydrothiophen-3-yl) methyl) ureido) hexyl) carbamate (peak **6**) was a piperidine alkaloid. Both compounds were identified for the first time, and peak **6** was found exclusively in the leaf. *M. koenigii* is a rich source of carbazole alkaloids [19]. Carbazole is a tricyclic heterocyclic molecule, which is a building block of natural alkaloids and possesses various potential biological activities, such as anticancer, anti-bacterial, and antiviral [20]. The diverse derivatives of carbazole have demonstrated antimicrobial activities, showcasing the potential of this compound in medicinal chemistry due to its outstanding drug-like properties and significant biological effects [21]. It has been identified from various plants of the genera *Murraya, Causena* and *Glycosmis*, all of which belong to the family Rutaceae [20].

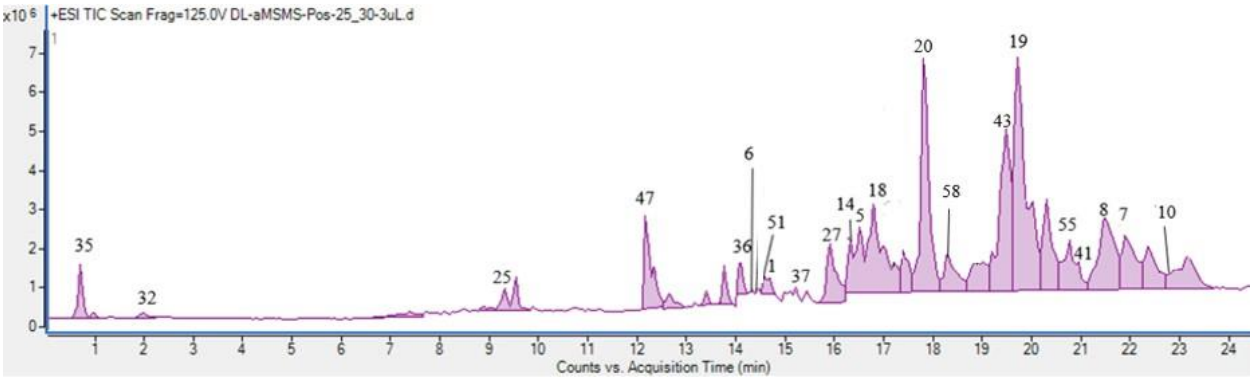
Piperidine is a 6-membered ring having 5-methylene spacer and one bridge of amine [22]. Research has focused on studying piperidine derivatives due to their potential in exhibiting a wide range of beneficial effects, including anticancer, antimalarial, anti-inflammatory, antifungal, antiviral, antimicrobial, antihypertensive, and anti-Alzheimer properties [23]. Piperidine alkaloids, which constitute a significant group of plant compounds, exhibit a wide range of structural frameworks and diverse biological activities. They are one of the major classes of alkaloids which demonstrate significant pharmacological properties. Its potent antioxidant properties arise from its ability to hinder or suppress free radicals [23]. Additionally, piperidine alkaloids have also been investigated for their potential anticonvulsant and antiarrhythmic effects [22, 24].

Peak **7** in cluster 2 was identified as 2-(3,5,7-tripyridin-2-ylcyclooctyl) pyridine, a known compound, while the remaining three compounds (peaks **8**, **9**, **10**) were unknown. Compounds **7**, **8**, and **10** were alkaloids and were found abundantly in the leaf of *M. koenigii*. In cluster 2, peak **7** (m/z 481.2991), peak **8** (m/z 483.3135), and peak **10** (m/z 481.2994) showed significant similarity in their parent ions. This suggested that these compounds shared commonalities in chemical or structural characteristics. However, peak **9** was identified as 1-(4-methyl-1,1-dioxidotetrahydrothiophen-3-yl)-3-(2,2,6,6-tetramethylpiperidin-4-yl) urea with a different parent ion (m/z 332.2014) was lacking similarity to the other compounds in the cluster. This observation prompted a deeper exploration of the underlying factors responsible for this clustering phenomenon. This compound was abundantly found in leaf (68%) as compared to the bark (28%) and root (5%). Leaf contains numerous chloroplasts, which is the vital organelles for plant photosynthesis. Plants require an adequate supply of nitrate (NO_3^{2-}) to stimulate leaf growth and photosynthesis. Given the critical role of nitrogen in supporting chloroplast and photo-synthetic functions, the identification of urea-derived compounds in leaves abundantly is highly plausible.

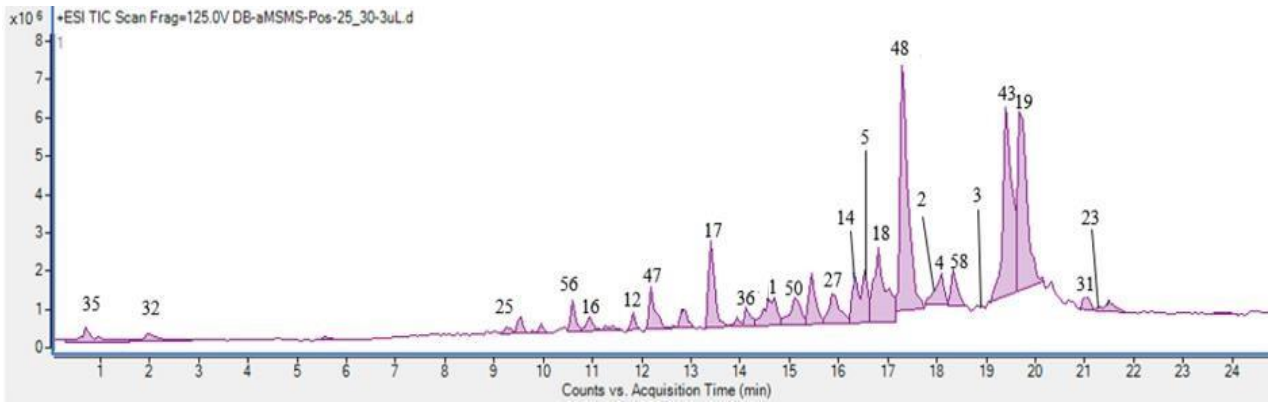
Most of the compounds annotated in cluster 3, 4 and 5 were known compounds, including 5,7-dihydro-6H-dibenzo[d,f][1,3]diazepin-6-one (peak **11**), 4-methyl-acridone (peak **12**), acridone (peak **13**), benzacridine (peak **14**), benz(b)acridine (peak **15**), 2-methoxy-10H-acridin-9-one (peak **17**), mahanimbine (peak **19**), and mahanine (peak **20**). Peaks **16** and **18** were putatively identified as methyl 1-methyl-9H-pyrido[3,4-b] indole-3-carboxylate and 4-methoxy- N-(4-methoxyphenyl)-N-(4-propoxyphenyl) aniline, respectively. To the best of our knowledge, these two compounds were putatively identified for the first time and hence their biological activities remain largely understudied.

In cluster 3, peak **11** with RT at 10.42 minutes and parent ion at m/z 211.0865 $[M+ H]^+$ dissociated to yield fragment ions at m/z 167.0725 and 166.0615 when C_2H_2O is eliminated. Peaks **12** (RT 11.83 and m/z 210.0915 $[M+ H]^+$) and **13** (RT 12.82 and m/z 196.0754 $[M+ H]^+$) yielded fragment ions of m/z 166.0615 and m/z 167.0722, after the loss of C_2H_3O and CO group, respectively. In addition, peaks **14** (RT 16.33) and **15** (RT 16.72), which shared the same molecular formula ($C_{17}H_{11}N$) and parent ion (m/z 248.1071 $[M + H_2O+H]^+$), yielded the same fragment ion (m/z 205.0865) by eliminating two carbon atoms.

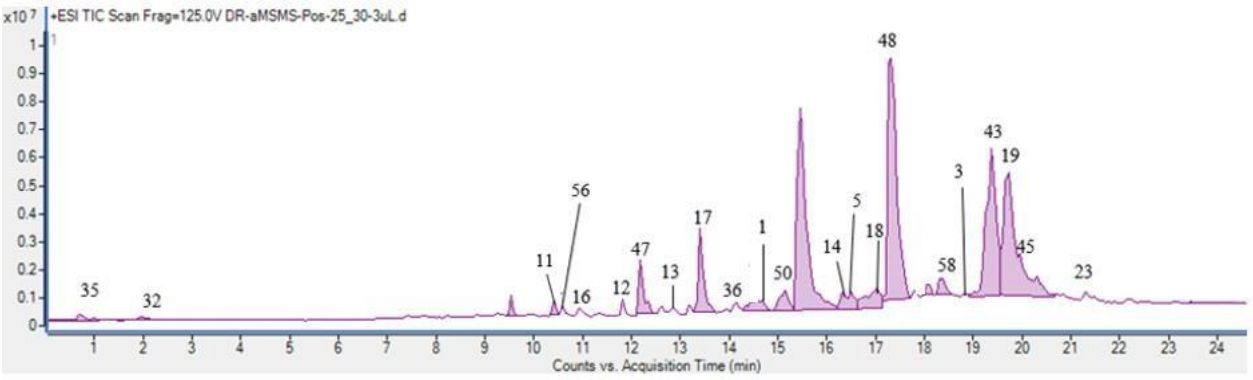
The co-clustering of all five compounds in cluster 3 can be primarily attributed to the presence of acridine-like moieties in 4-methyl-acridone, acridone, benzacridine, and benza(b)cridine. Notably, 5,7-dihydro-6H-dibenzo[d,f][1,3]diazepin-6-one has a different structural framework, characterized by the complex heterocyclic diazapinone ring. However, despite this structural difference, it exhibits similar MS/MS fragment ions (m/z 168.0808), 4-methyl-acridone (m/z 167.0730) and acridone (m/z 168.0808). This shared spectral feature led to the inclusion of 5,7-dihydro-6H-dibenzo[d,f][1,3]diazepin-6-one in the same cluster as other compounds with acridine-like structures.



(a)



(b)



(c)

Figure 2. Total ion chromatogram (TIC) in positive ion mode of the (a) leaf, (b) bark and (c) root extracts of *Murraya koenigii*. The number above each peak represents the peak numbers, corresponding to the peak numbers in Table 1.

Other than that, peak **16** in cluster 4 displayed a $[M + H]^+$ ion at m/z 241.0972 with the formula $C_{14}H_{12}N_2O_2$, and it exhibited characteristic fragment ions at m/z 198.0921, 183.0679, 167.0730, and 154.0651 by indicating successive losses of C_2H_2O, CH_3, an oxygen atom and CH, respectively. Peak **17** (m/z 226.0865 $[M + H]^+$) with the molecular formula $C_{11}H_{17}NO_2$ yielded fragment ions by losing C_2H_3 (m/z 198.0861), NH_2 (m/z 183.0658), an oxygen atom (m/z 167.0715) and CO_2 (m/z 154.0654). Despite peak **17** was having an acridine-like structure, it was not categorised within cluster 3, which might have been expected based on structural similarities. The unique aspect of cluster 4 lies in the fact that both compounds within it, peak **16** and **17**, shared similar MS/MS fragmentation ions. This shared spectral feature appeared to have played a key role in the compounds' clustering within cluster 4, distinguishing them from compounds in cluster 3. Cluster 5 consisted of three carbazole alkaloids with similar molecular formulas ($C_{23}H_{25}NO_3$, $C_{23}H_{25}NO$, and $C_{23}H_{25}NO_2$). They were grouped together because they shared a common carbazole core structure, even though they have slight differences in the number of oxygen atoms.

Only four compounds from the pool of the known compounds in the MN have been previously isolated from *M. koenigii*, which were mahanimbine, mahanine, girinimbine and caryophyllene oxide. Mahanimbine (peak **19**), with the parent ion of m/z 332.2013 $[M + H]^+$, is a carbazole alkaloid previously found in the leaves, roots, seeds and fruits of *M. koenigii* [13]. Other than that, it has also been isolated from other plant species under the genus *Murraya*, such as the root bark of *M. euchrestifolia*, [25], the flowers, leaves, and twigs of *M. siamensis* [26], and the leaves of *M. paniculata (L.) Jack* [27]. In terms of pharmacological activity, mahanimbine acts as a potential antihyperlipidemia [13,27], antioxidant, antimicrobial, antidiabetic [13,28], and anticancer agent. Mahanine (peak **20**) with parent ion m/z 332.2013 [M + H] is also a carbazole alkaloid found in the leaves of *M. koenigii* [3]. It has been found to possess anticancer [29], antimutagenic, antileishmanial [30] and antimicrobial properties [31]. Peak **48**, which identified as girinimbine m/z 264.1390 $[M + H]^+$, is a carbazole alkaloids with potential therapeutic properties, has been identified from the root, barks, and seeds of *M. koenigii* [13]. It displays anticancer and anti-inflammatory actions [32], as well as *in vitro* anti-tumour promoting, strong antioxidant [33] and gastro-protective activity. Caryophyllene oxide (peak **42**), with parent ion m/z 221.1896 $[M + H]^+$ was identified as an epoxide which was a minor metabolite found in the essential oils of *M. koenigii* leaves [10]. It demonstrates anti-inflammatory, antioxidant, antiviral, anticarcinogenic, and analgesic properties.

Cluster 6 consisted of two compounds, which were identified as 1-(2-hydroxypropan-2-yl)-4,7-dimethyl-1,3,4,5,6,8a-hexahydronaphthalen-4a(2H)-ol (peak **21**) and bisabolol oxide A (peak **22**). Peak **21** was identified for the first time from *M. koenigii* and no prior report. Based on the m/z value of MS/MS fragment ions for compounds in Cluster 6, peak **21** with parent ion m/z 348.1965 $[M - H_2O + H]^+$ and molecular formula $C_{15}H_{26}O_2$, yielded fragment ion at m/z 203.0534 by eliminating both OH and CH_3 groups. Another compound in same cluster, peak **22** with same parent ion and molecular formula as peak **21** yielded fragment ion at m/z 203.1794 by losses of H_5O group. These two compounds have the same expected molecular weight (238.3657 m/z) and produced a matching precursor ion at m/z 221.1901, further enhancing the similarity of their structures. Furthermore, their MS/MS fragmentation consistently produced fragment ions at m/z 203.1794. These similarities highlighted the strong structural connections between these compounds, which supported their aggregation in the same molecular network. Bisabolol oxide A belongs to the sesquiterpene class and exists as a major compound in various plants within the Asteraceae and Labiatae families, including *Chamomilla recutita* (L.) Rauschert [24], *Matricaria chamomilla* (*Matricaria recutita*) [34,35,36], *Chamaemalum nobile* and *Thymus capitatus* [37]. Bisabolol oxide A serves as one of the main biologically active compounds present in the chamomile oil.

1-(2-hydroxypropan-2-yl)-4,7-dimethyl-1,3,4,5,6,8a-hexahydronaphthalen-4a(2H)-ol (peak **21**), bisabolol oxide A (peak **22**), and 1,4,4,8-tetramethyl-12-oxatricyclo [6.3.1.02,5] dodecan-9-ol (peak **34**) belonged to the same terpenoid class. They shared identical molecular formula ($C_{15}H_{26}O_2$) and very similar parent ion masses (m/z 221.1900, m/z 221.1901, and m/z 221.1901, respectively), suggesting that they belong to the same cluster. The present study showed that peaks **21** and **22** were indeed clustered together, specifically in cluster 6 but peak **34** stood alone as a single node, contrary to the expected cluster placement. This discrepancy can be attributed to differences in the compounds' polarity. This contrast in polarity was clearly exemplified by the findings of reverse-phase chromatography. Peak **34** exhibited an elution time of 12.23 minutes, while peaks **21** and **22** eluted at 17.39 and 17.21 minutes, respectively. Considering the reversed- phase chromatography, it can be concluded that peak **34** is the most polar compound among all the three compounds. This characteristic set it apart from peaks **21** and **22**, justifying its unique cluster assignment in the study.

The remaining compounds located on the single nodes are known compounds from the fatty acids, terpenoids and alkaloids classes. Fatty acids are primary metabolites and was postulated that they might not possess any pharmacological effects, hence were not discussed further in this study. Terpenoids are secondary metabolites and have been shown to possess biological activities in some studies [38]. However, the information about 1,4,4,8-tetramethyl-12-oxatricyclo [6.3.1.02,5] dodecan-9-ol (peak **34**), and (8R,10S,13S)-13-methyl-1,2,5,6,7,8,9,10,11,12,

14,15,16,17-tetradecahydrocyclopenta[a]phenanthren-17-ol (peak **40**) is lacking. Despite an extensive search through scholarly sources and the existing literature, we could not find any prior reports or references to other single nodes.

In this study, molecular networking provided an expedited route in the dereplication of known compounds in the crude extracts of *M. koenigii*. The clustering of the compounds in clusters 3, 4 and 6 of the MN was based on the similarity in their MS/MS fragmentation patterns, while for clusters 1, 2 and 5 were clustered according to their molecular classes. Consequently, a total of 11 potential new compounds were predicted putatively from the MN clusters due to their structure similarities. This not only accelerated the discovery of potential new compounds but also offered insight into the distribution of each compound in different parts of the *M. koenigii* plant. To illustrate, among the 11 potential new compounds, certain substances were exclusively found in the bark (peaks 2 and 4), some were solely present in the leaves (peak 6), while others were distributed across the leaves, bark, and roots. The findings of the present study suggested that the alkaloid compounds in the bark and root of *M. koenigii* might appear to be potential bioactive compounds deserving of further investigation.

CONCLUSION

In a nutshell, molecular networking serves as a good dereplication strategy in dereplicating known compounds from a complex mixture. The present study suggested that the alkaloid compounds in the bark and root of *M. koenigii* might appear to be potential bioactive compounds. The new alkaloid compounds, (1-(4-ethoxybutyl)-2-methyl-1H-indol-3-yl) (p-tolyl) methanone (peak **2**), and 1,4-bis(3-phenyl propyl)-1,2-dihydropyridine (peak **4**), which were putatively identified in the bark whereas 2-methyl-4,6-bis(4-(pyridin-4-yl) phenyl)-1,3,5-triazin (peak **3**), which was found abundantly in the root of *M. koenigii* could be the potential candidates for future research, including targeted compound isolation, followed by structural elucidation and bioassays, to be further explored as a potential lead compound in drug discovery.

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