Current Research in Complementary & Alternative Medicine
Kalaichelvam RK, et al. Curr Res Complement Altern Med 6: 164.
www.doi.org/10.29011/2577-2201.100064
www.gavinpublishers.com

Research Article

Comprehensive Metabolite Profiling of 50% Ethanol-Aqueous *Clinacanthus nutans* Leaf Extraction by using GC-MS and LC-MS

Ravi Kumar Kalaichelvam, Vuanghao Lim, Ida Shazrina Ismail*

Advanced Medical and Dental Institute (IPPT), Universiti Sains Malaysia, Bertam, 13200 Kepala Batas, Penang, Malaysia

***Corresponding Author:** Ida Shazrina Ismail, Regenerative Medicine Cluster, Advanced Medical and Dental Institute (IPPT), Universiti Sains Malaysia (USM), 13200, Bertam, Pulau Pinang, Malaysia

Citation: Kalaichelvam RK, Lim V, Ismail IS (2022) Comprehensive Metabolite Profiling of 50% Ethanol-Aqueous *Clinacanthus nutans* Leaf Extraction by using GC-MS and LC-MS. Curr Res Cmpl Alt Med 6: 164. DOI: 10.29011/2577-2201.100064

Received Date: 11 November 2022; **Accepted Date:** 21 November 2022; **Published Date:** 25 November 2022

Abstract

Clinacanthus nutans (Burm f.) Lindau (C. nutans) or commonly known as belalai gajah or Sabah snake grass, is a traditionally well-known traditional medicine in Southeast Asia. It consists of abundant phytomedicinal properties such as anti-viral, anti-bacterial and anti-inflammation. This work aims to profile the phytochemical compounds in 50% ethanolic (EtOH-Aq) extract of *C. nutans* leaves and tentative identification for future study of the immunomodulatory activities. Crude extract was obtained from the dried leaves through the maceration of 50% ethanol. The resultant extracts were investigated for metabolites profiling using the gas chromatography mass spectrometry (GC-MS) and accurate-mass QTOF liquid chromatography-mass spectrometry/Mass spectrometry (LC-MS/MS). There were more than 100 compounds profiled by both methods. Both chromatography methods exhibited a good source of phenolics, flavonoids, triterpenoids, steroids, phytosterols, and glycosides in *C. nutans*. Vitexin, isovitexin, isoorientin, riboflavin, succinate and luteolin were among the compounds that have been tentatively identified. 5 compounds present in both positive and negative LC-MS/MS ion mode which are isovitexin 2''-O-xyloside, 6,8-Di-C-beta-D-arabinopyranosyl apigenin, isovitexin5'-S-Methyl-5'-thioinosine, and alliin. This study provides informative data on the potential metabolites of 50% ethanol- aqueous *C. nutans* extract for future studies.

Keywords: *Clinacanthus nutans*; GC-MS; LC-MS; Ethanolic extract; Metabolites

Introduction

In order to meet health care needs, almost 80% of the world populations depends on traditional practitioners and their armamentarium of medicinal plants and herbs [55]. There is more than 2000 plant species that have medicinal properties and highly potential to be commercialised [44]. Nowadays, many herb properties have been well documented due to the increasing interest and research effort for their applications in pharmaceutical, nutritional and cosmetic application.

Clinacanthus nutans (*C. nutans*) is well known as Sabah snake grass which belongs to the *Acanthaceae* family. This plant has diverse and potential medicinal uses in traditional herbal medicine for treating skin rashes, insects and snake bites, lesions caused by herpes simplex virus, diabetes, and gout [39]. Extracts from the leaves of *C. nutans* have been identified to possess analgesic, anti-inflammatory, and antiviral activities against Varicella-zoster virus (VZV) and Herpes simplex virus (HSV) -2 [8]. *C. nutans* has been phytochemically and chemically investigated previously for stigmasterol, lupeol, β-sitosterol belutin [13]. Six known C-glycosyl flavones, vitaxin, isovitexin, shaftoside, isomollupentin-7-O-βglucopyranoside, orientin, isoorientin, five sulfur-containing glycosides, two glycoglycerolipids, a mixture

of nine cerebroside and a monoacylmonogalatosylglycerol, have been isolated [50]. Extracts from *C. nutans* have been used to reduce symptoms of inflammation in insect bites, Herpes infection and allergic responses in traditional medicine. A few reports have also described the effects of *C. nutans* extracts on the immune system. *C. nutans* could modulate the adaptive immune system by skewing the immune system response and also to act on the innate immune system [24].

Plants are rich in active compounds or metabolites such as alkaloids, steroids, tannins, glycosides, volatile oils, fixed oils, resins, phenols and flavonoids which can be found in each parts of the plants such as leaves, flowers, bark, seeds, fruits, and root. Metabolites are small biomolecules that considered as non-essential for the life of the producer organism [1]. The metabolites provide survival advantages in the producer organism in various ways, for instance by improving nutrient availability in the form of chelating agents, by protecting against environmental stressors such as pigments and osmoprotectants, by enhancing competitive interactions with other organisms such as antibiotics and also various signalling molecules, or by acting as a metabolic defence mechanism [9].

There have two type of metabolites which are primary and secondary metabolites. Plants contain both primary and secondary metabolites. There are differences between both types of metabolites. Primary metabolites are chemical constituents that are naturally present in most of the plant and directly involved in the plant growth [17]. Meanwhile, secondary metabolites are the bioactive phytochemicals which are normally produced by a plant in response to specific environmental stresses and considered to possess additional health benefits to the respective plants [17]. Phytochemical screening tests, thin layer chromatography (TLC) and Fourier transform infrared spectroscopy (FTIR) are the most popular method used to identify the metabolites presence in a plant. This is because these methods are easy to conduct, low cost and time effective. Guidelines on the class and functional groups of the chemical constituents that are present in plant can be obtained by using these methods [46].

In this research, the extracted metabolites from 50% ethanolic (EtOH-Aq) *C. nutans* extract were detected by using gas chromatography-mass spectrometry (GC-MS) and liquid chromatography- tandem mass spectrometry (LC-MS/MS). GC-MS and LC-MS/MS are widely used as a tool for metabolite identification in metabolomics. GC-MS provides high sensitivity, good separation, low cost and user-friendly [27]. GC-MS technique is a fast and accurate method which has been applied in diagnostics, screening and functional genomics purposes due to its ability to characterise hundreds of metabolites from different chemical groups in one analytical run [43]. GC-MS is an integral tool due to its ability to produce reproducible molecular fragmentation patterns which is very suitable for metabolite identification. LC-MS involves separating mixtures in accordance with their physical and chemical properties, then identifying the components within each peak and detecting based on their mass spectrum. The flow rates used in LC-MS should be less than those used for HPLC. This is to ensure complete ionisation and to maintain the detection sensitivity of the MS.

The type of *C. nutans* extract used in this study was based on the research carried by our team previously to identify the potential extract of *C. nutans* which was 100% ethanolic and 50% EtOH-Aq extract of *C. nutans* leaves with the J774.2 macrophage cell line. Based on the findings of the study, 50% EtOH-Aq extract was chosen for this study, which demonstrates potential immunomodulation in the macrophage cell line [22]. In this study, we used a 50% EtOH-Aq extract to look for metabolites in the *C. nutans* leaves extract sample. GC-MS and LC-MS/MS analyses was performed to profile the metabolites present in the 50% EtOH-Aq *C. nutans* extract.

Method

Collection and extraction of *C. nutans*

C. nutans was harvested from Manjung, Perak, Malaysia. The botanical identification of the plants was determined by the Herbarium Unit, School of Biological Sciences, Universiti Sains Malaysia, Penang (Voucher no.: USM Herbarium 11465). The leaves were separated from the stem, cleansed and oven dried at 40 - 45 °C for 5-7 days prior to pulverisation into fine powder.

The readily dried leaves of *C. nutans* was pulverised into fine powder using herb grinder (Retsch, Haan, Germany). The powdered leave was mixed separately with the solvent which was 50% EtOH by applying a solid to solvent ratio of 1:15 (w/v). 10g of the powdered leaves was mixed with 150mL of solvent in a conical flask and the mixtures was subjected to ultrasonic assisted shaking process for 30 min using ultrasonic cleaner bath (PMI Labortechnik, Grafstal, Switzerland). After that, the mixture was centrifuged at 1250*g* for 15 minutes. A vacuum filtration (Vacuubrand, Wertheim, Germany) method was used to filter the obtained supernatant. Then, the residue obtained from the previous filtration process was re-used to repeat the entire extraction process twice. The extract proceeded to freeze dry by using Freeze dryer (Alpha 1-4 CSC RVC 2-25). Then, the extract was tightly sealed and kept at 4 ˚C until further use.

The percentage yield of the extracts was calculated using the following equation:

Percentage yield= [Weight of extract (g) ÷ Weight of leaves (g)] × 100 %

Derivatisation of *C. nutans* extracts for GC-MS

Derivatisation process was carried out by mixing 5 mg of *C. nutans* extract with 50µL of pyridine and 75 µL of bis(trimethysilyl) trifluoracetamine (BSTFA). The mixture was then heated for 20 minutes at 80 ˚C in the sonicator. The derivatised samples were filtered using a 0.45 µm nylon membrane filter to remove any suspended particulate particle residue. The sample was then transferred to micro volume vial insert for GC-MS analysis [20].

GC-MS analysis of *C. nutans*

The GC-MS analysis was carried out using the Agilent Technologies 7890 A, New York, US. An aliquot (2 µL) of the derivatised sample was injected into the gas chromatography at a split ratio 1:20 [20]. The carrier gas used was helium with flow rate of 1 mL/min. The injector was operated at 200°C with oven temperature programmed from 100 ˚C to 300 ˚C at the rate of 1.2 °C/min [20]. Identification of the compounds was conducted according to National Institute of Standards and Technology/ Gaithersburg MD USA (NIST MS) search 2.0 [41]. Each compound was identified by relating their peak retention time and peak area (%).

LC/Q-TOF-MS/MS analysis of *C. nutans*

Agilent 1290 Infinity LC system coupled to Agilent 6520 Accurate-Mass Q-TOF mass spectrometer with dual ESI source was used to perform the LC-MS/MS analyses. The LC separation was conducted on Agilent Zorbax Eclipse XDB-C18, Narrow-Bore 2.1x150mm, 3.5 micron (P/N: 930990-902) with an injection volume of 1µL and a column temperature of 25 ˚C. The mobile phases consisted of A which was 0.1% formic acid in water and B which was 0.1% formic acid in acetonitrile at a flow rate of 0.5mL/ mins. Elution was programmed as a linear gradient that began by increasing from 5 to 100% B in 25 min, held at 100% B for 3 min, decreasing from 100 to 5% B in 1 min, and then maintained at 5% B for 2 min. Mass spectra were generated in both negative and positive ion mode with an electrospray ionisation (ESI) source. Nitrogen was used as the drying gas at 300 ˚C and the ESI spray voltage was 4000 V. Full-scan mass spectra were acquired over an m/z range of 100 to 3200. Data was processed with Agilent MassHunter Qualitative Analysis B.07.00. To identify the metabolites, Metlin_AM_PCDL-N-170502.cdb database was used with match tolerance 5ppm and the data compared with MS DIAL software data.

Result

The percentage yield of the *C. nutans* extracts was calculated by using the formula stated above. In this experiment, 20g of plant sample was used with 300mL of solvent. The percentage yield from 20g of sample was 12.1%. The EtOH-Aq obtained from freeze-drying was in golden brown colour. The extracts appeared in crystal like powder textures.

The active metabolites of 50% EtOH-Aq *C. nutans* extraction were analysed using GC-MS **(Figure1)** and LC-MS/MS **(Figure 2 and 3)**. In both the GC-MS and the LC-MS/MS analyses, prominent peaks were identified. To identify the nature and structure of the compounds, the mass spectrometer analysed the compounds eluted at different times. The large compound fragments into small compounds, causing peaks with different m/z ratios to appear. These mass spectra are the compound's fingerprint, which can be identified using the data library.

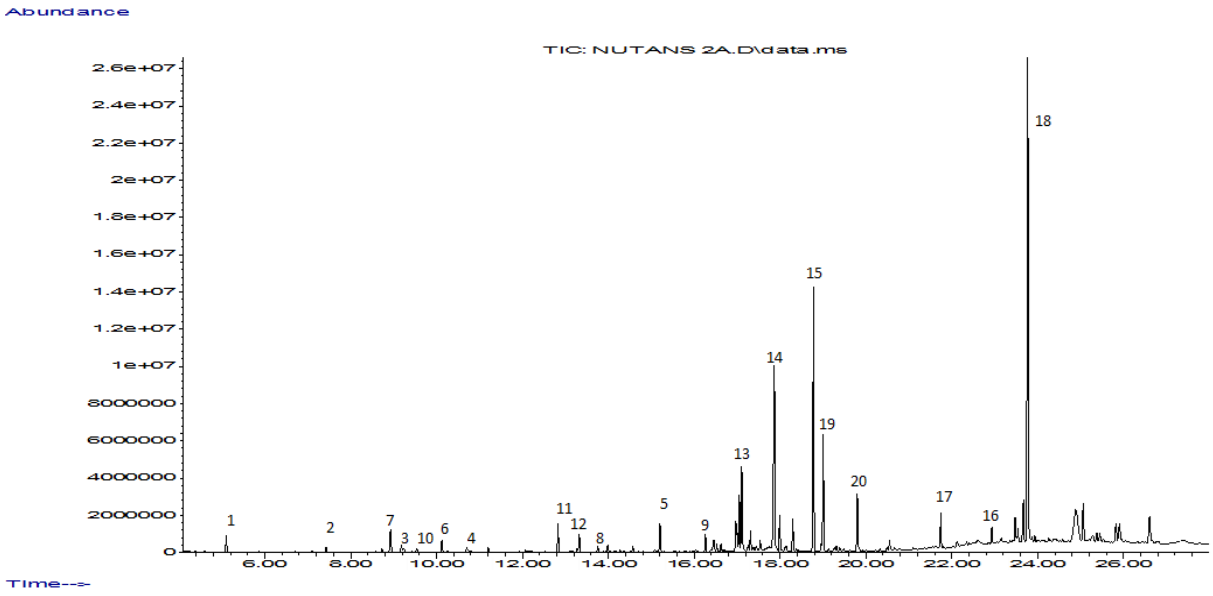


Figure 1: Total Ion Chromatogram (TIC) spectrum of GC-MS analysis by using 50% EtOH-Aq *C. nutans* extraction. Total 20 compounds identified from GC-MS analysis. Identified compounds:- L-Alanine(1), L-Valine(2), L-Proline(3), L-threonine(4), L-Asparagine(5), Propanoic acid(6), Glycerol(7), Trihydroxybutyric acid(8), 2-Keto-l-gluconic acid(9), Butanedioic acid(10), Malic acid(11), Butanoic acid(12), D-Galactose(13), beta.-D-Glucopyranose(14), D-Glucose(15), D-Glucose(16), alpha.-d-galactopyranoside(17), Maltose(18), Galactonic acid(19), Myo-Inositol(20).

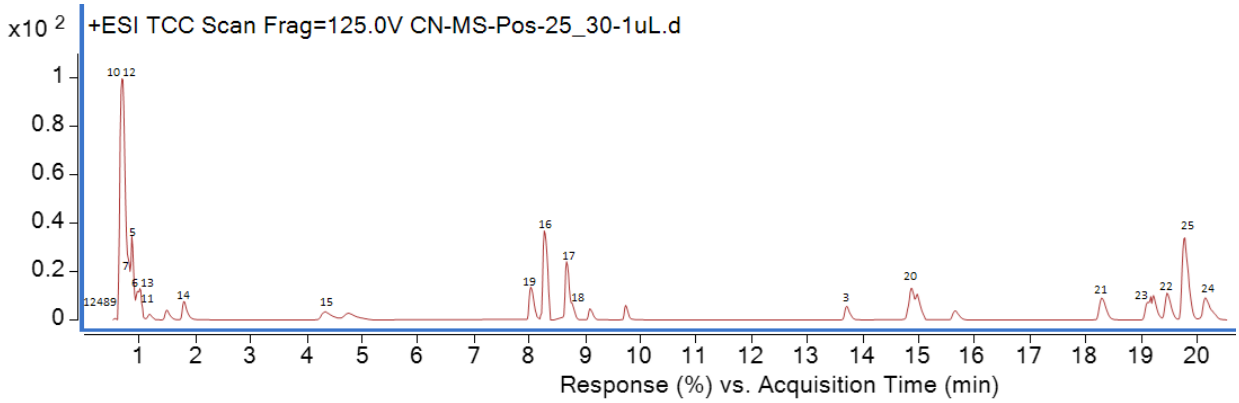


Figure 2 TIC spectrum of LC-MS/MS positive ion analysis by using 50% EtOH-Aq *C. nutans* extract. Total 25 compounds identified from positive ion mode LC-MS/MS analysis. Identified compounds:- N-Ethylpropylamine(1), Artemisinin(2), (S)-Nerolidol 3-O-[a-L-Rhamnopyranosyl-(1->4)-a-L-rhamnopyranosyl-(1->2)-b-D-glucopyranoside](3), D-Norvaline(4), 4-Mercaptobutyric acid(5), Alliin(6), 3-cyclopentyl-DL-alanine(7), Carisoprodol(8), 1-Aminocyclohexanecarboxylic acid(9), Adenine(10), Tyrosine(11), 2-dec sulfonyl thiomide(12), 5'-S-Methyl-5'-thioinosine(13), Gentiatibetine(14), 2-Propenoic acid, 3-(1H-indol-3-yl)(15), Coumermic acid(16), 6,8-Di-C-beta-D-arabinopyranosyl apigenin(17), Isovitexin(18), Isovitexin 2''-O-xyloside(19), 5Z,8Z,11Z,14Z-Octadecatetraenoic acid(20), Pheophorbide b(21), Pheophorbide a(22), Harderoporphyrin(23), Oligomycin A(24), Betulinic acid(25)

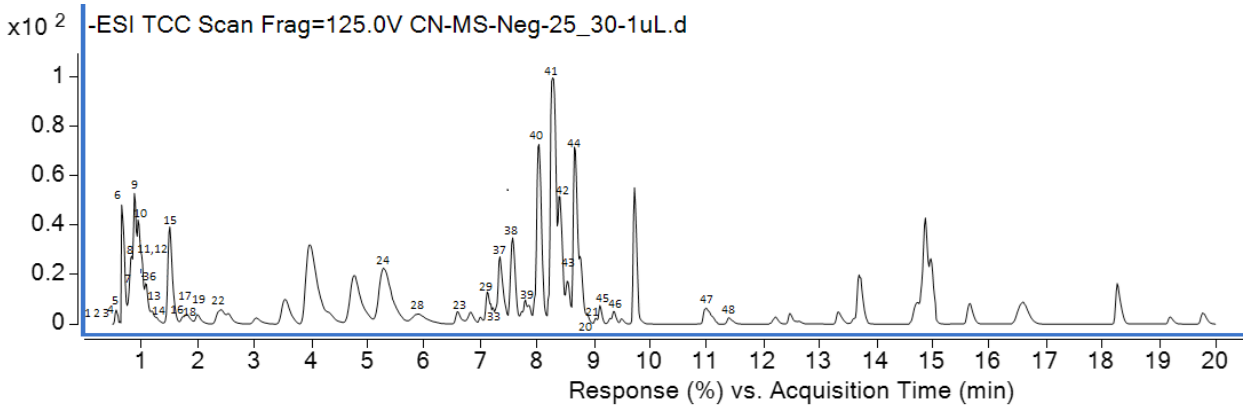


Figure 3 TIC spectrum of LC-MS/MS negative ion analysis by using 50% EtOH-Aq *C. nutans* extract. Total 48 compounds identified from negative ion mode LC-MS/MS analysis. Identified compounds are:- L-Threonic acid(1), Dictyoquinazol C(2), 1-Hydroxyanthraquinone(3), Pyrazole(4), Clotiazepam(5), Purpuritenin A(6), Dyphylline(7), 5'-S-Methyl-5'-thioinosine(8), Alliin(9), 3-Amino-3-(4-hydroxyphenyl)propanoic acid(10), Topiramate(11), S-Inosyl-L-homocysteine(12), N-(1-Deoxy-1-fructosyl) leucine(13), Guanosine(14), Galloylglycerol(15), Hetacillin(16), N-(1-Deoxy-1-fructosyl)phenylalanine(17), Benzocaine(18), Geniposidic acid(19), Schizonepetoside E(20), Neryl rhamnosyl-glucoside(21), 2-O-Caffeoylglucaric acid(22), Loganic acid(23), Caffeic acid 3-O-glucuronide(24), Ferulic acid 4-O-glucuronide(25), Ethotoin(26), Riboflavin(27), 2-(E)-O-Feruloyl-D-galactaric acid(28), Acetylaminodantrolene(29), Arjunolic acid(30), Methylgingerol(31), Pheophorbide a(32), Feruloyl arabinobiose(33), Isoferuloyl C1-glucuronide(34), Dihydroalbocycline(35), Cerarvensin(36), Isoorientin 7-O-glucoside(37), Robinetin 3-rutinoside(38), Luteolin 7-robinobioside(39), Quercetin 3-(2-caffeoylsophoroside) 7-glucoside(40), Isovitexin 2''-O-xyloside(41), 6-Hydroxyluteolin 5-rhamnoside(42), 6,8-Di-C-beta-D-arabinopyranosyl apigenin(43), Hexaacetylpyracanthoside(44), Isovitexin(45), Nonic Acid(46), , 11-Hydroperoxy-12,13-epoxy-9-octadecenoic acid(47), 5,8,12-Trihydroxy-9-octadecenoic acid(48)

In LC-MS/MS analysis, a total of 25 and 49 metabolites were identified through positive and negative ion modes, respectively, and tabulated in **Tables 1 and 2**. In this research, mixture of 2 types solvent which is water (alkali) and ethanol (acid) was used to extract the metabolites. Basic metabolite will have positive charge which can be found in water and it will be observed in positive mode. While acid metabolite will have negative charge and can be observed in negative mode [32]. From the analysis, more acid metabolites compared to basic metabolites were present in 50% EtOH-Aq *C. nutans* extraction.

No	Rt	Mass	Name	Molecular formula	Class
1	0.626	87.16	N-Ethylpropylamine	$C_5H_{13}N$	Primary Amine
2	0.662	234.16	Artemisinin	$C_{15}H_{22}O_2$	Sesquiterpenoid
3	13.71	676.37	(S)-Nerolidol 3-O-[a-L-Rhamnopyranosyl-(1->4)-a-L-rhamnopyranosyl-(1->2)-b-D-glucopyranoside]	$C_{33}H_{56}O_{14}$	
4	0.664	117.08	D-Norvaline	$C_5H_{11}NO_2$	Alpha amino acids
5	0.864	120.03	4-Mercaptobutyric acid	$C_4H_8O_2S$	
6	0.985	177.05	Alliin	$C_6H_{11}NO_3S$	
7	0.791	157.11	3-cyclopentyl-DL-alanine	$C_8H_{15}NO_2$	
8	0.690	260.17	Carisoprodol	$C_{12}H_{24}N_2O_4$	Carbamate
9	0.706	143.09	1-Aminocyclohexanecarboxylic acid	$C_7H_{13}NO_2$	Monoterpenoids
10	0.725	135.05	Adenine	$C_5H_5N_5$	Purine
11	1.003	181.07	Tyrosine	$C_9H_{11}NO_3$	

12	0.736	279.13	2-dec sulfonyl thiomide	$C_{12}H_{25}NO_2S_2$	Pyridines
13	0.946	298.07	5'-S-Methyl-5'-thioinosine	$C_{11}H_{14}N_4O_4S$	Inosine
14	1.800	165.08	Gentiatibetine	$C_9H_{11}NO_2$	Alkaloid
15	4.348	187.07	2-Propenoic acid, 3-(1H-indol-3-yl)	$C_{11}H_9NO_2$	Phenylpropanoids
16	8.281	547.12	Coumermic acid	$C_{27}H_{21}N_3O_{10}$	
17	8.678	534.13	6,8-Di-C-beta-D-arabinopyranosyl apigenin	$C_{25}H_{26}O_{13}$	Flavonoids
18	8.778	432.11	Isovitexin	$C_{21}H_{20}O_{10}$	
19	8.068	564.15	Isovitexin 2''-O-xyloside	$C_{26}H_{28}O_{14}$	
20	14.985	276.21	5Z,8Z,11Z,14Z-Octadecatetraenoic acid	$C_{18}H_{28}O_2$	Linoleic acid
21	18.317	606.25	Pheophorbide b	$C_{35}H_{34}N_4O_6$	Chlorins
22	19.783	592.27	Pheophorbide a	$C_{35}H_{36}N_4O_5$	
23	19.131	608.27	Harderoporphyrin	$C_{35}H_{36}N_4O_6$	Porphyrins
24	20.244	790.52	Oligomycin A	$C_{45}H_{74}O_{11}$	Macrolide
25	19.783	456.70	Betulinic acid	$C_{30}H_{48}O_3$	Triterpenoid

Table 1: List of compounds found in 50% EtOH-Aq C. nutans extract through LC-MS/MS positive ion mode which shows Retention time (Rt) for each of the compounds. There is total 24 compounds shortlisted. The compounds were sorted by look on the Score Db and Score MFG that are closed to 100. From all the compounds with high scores obtained, the compounds with Diff (Db.ppm) and Diff (MFG.ppm) that has value within -2 to +2 are selected. All the 24 compounds categorized into their own organic class.

No	Rt	Mass	Name	Molecular formula	Class
1	0.659	136.04	L-Threonic acid	$C_4H_8O_5$	Sugar acids
2	0.662	342.12	Dictyoquinazol C	$C_{18}H_{18}N_2O_5$	Quinazolines
3	0.685	224.05	1-Hydroxyanthraquinone	$C_{14}H_8O_3$	Anthraquinone
4	0.797	68.04	Pyrazole	$C_3H_4N_2$	Azoles
5	0.816	318.06	Clotiazepam	$C_{16}H_{15}ClN_2O\ S$	Thienodiazepines
6	0.818	292.11	Purpuritenin A	$C_{19}H_{16}O3$	Flavonone
7	0.879	254.10	Dyphylline	$C_{10}H_{14}N_4O_4$	Xanthines
8	0.954	298.08	5'-S-Methyl-5'-thioinosine	$C_{11}H_{14}N_4O_4S$	Inosine
9	0.983	177.05	Alliin	$C_6H_{11}NO_3S$	Alpha amino acid
10	1.009	181.07	3-Amino-3-(4-hydroxyphenyl) propanoic acid	$C_9H_{11}NO_3$	Beta amino acids
11	1.014	339.10	Topiramate	$C_{12}H_{21}NO_8S$	Dioxolopyrans
12	1.014	385.11	S-Inosyl-L-homocysteine	$C_{14}H_{19}N_5O_6S$	Organosulfonic acids
13	1.023	293.15	N-(1-Deoxy-1-fructosyl) leucine	$C_{12}H_{23}NO_7$	Leucine
14	1.197	283.09	Guanosine	$C_{10}H_{13}N_5O_5$	Purine
15	1.455	244.06	Galloylglycerol	$C_{10}H_{12}O_7$	Gallic acid
16	1.507	389.14	Hetacillin	$C_{19}H_{23}N_30_4S$	Penicillins
17	1.746	327.13	N-(1-Deoxy-1-fructosyl) phenylalanine	$C_{15}H_{21}NO_7$	Phenylalanine
18	1.811	165.08	Benzocaine	$C_9H_{11}NO_2$	Benzoic acid esters

19	2.004	374.12	Geniposidic acid	$C_{16}H_{22}O_{10}$	Terpene
20	9.039	348.18	Schizonepetoside E	$C_{16}H_{28}O_8$	
21	9.368	462.25	Neryl rhamnosyl-glucoside	$C_{22}H_{38}O_{10}$	
22	2.374	372.07	2-O-Caffeoylglucaric acid	$C_{15}H_{16}O_{11}$	Iridoid
23	6.844	376.14	Loganic acid	$C_{16}H_{24}O_{10}$	
24	5.305	356.07	Caffeic acid 3-O-glucuronide	$C_{15}H_{16}O_{10}$	Phenolic
25	7.349	370.09	Ferulic acid 4-O-glucuronide	$C_{16}H_{18}O_{10}$	
26	4.371	204.09	Ethotoin	$C_{11}H_{12}N_2O_2$	Azolidines
27	4.778	376.14	Riboflavin	$C_{17}H_{20}N_4O_6$	Flavins
28	5.904	386.09	2-(E)-O-Feruloyl-D-galactaric acid	$C_{16}H_{18}O_{11}$	Indoles
29	7.221	326.10	Acetylaminodantrolene	$C_{16}H_{14}N_4O_4$	Hydantoin
30	13.335	488.35	Arjunolic acid	$C_{30}H_{48}O_5$	Triterpenoids
31	12.477	308.20	Methylgingerol	$C_{18}H_{28}O_4$	Phenol
32	19.782	592.27	Pheophorbide a	$C_{35}H_{36}N_4O_5$	Chlorins
33	7.456	458.14	Feruloyl arabinobiose	$C_{20}H_{26}O_{12}$	Phenylpropanoids
34	8.006	370.09	Isoferuloyl C1-glucuronide	$C_{16}H_{18}O_{10}$	
35	12.215	310.21	Dihydroalbocycline	$C_{18}H_{30}O_4$	
36	1.018	402.10	Cerarvensin	$C_{20}H_{18}O_9$	Flavonoids
37	7.580	580.14	Isoorientin 7-O-glucoside	$C_{26}H_{28}O_{15}$	
38	7.873	610.15	Robinetin 3-rutinoside	$C_{27}H_{30}O_{16}$	
39	7.950	594.16	Luteolin 7-robinobioside	$C_{27}H_{30}O_{15}$	
40	8.011	950.23	Quercetin 3-(2-caffeoylsophoroside) 7-glucoside	$C_{42}H_{46}O_{25}$	
41	8.283	564.15	Isovitexin 2''-O-xyloside	$C_{26}H_{28}O_{14}$	
42	8.346	448.10	6-Hydroxyluteolin 5-rhamnoside	$C_{21}H_{20}O_{11}$	
43	8.389	534.14	6,8-Di-C-beta-D-arabinopyranosyl apigenin	$C_{25}H_{26}O_{13}$	
44	8.774	702.18	Hexaacetylpyracanthoside	$C_{33}H_{34}O_{17}$	
45	9.357	432.11	Isovitexin	$C_{21}H_{20}O_{10}$	
46	9.498	188.11	Nonic Acid	$C_9H_{16}O_4$	
47	11.001	328.21	11-Hydroperoxy-12,13-epoxy-9-octadecenoic acid	$C_{18}H_{32}O_5$	Lineolic acids
48	11.401	330.24	5,8,12-Trihydroxy-9-octadecenoic acid	$C_{18}H_{34}O_5$	

Table 2: List of compounds found in 50% EtOH-Aq *C. nutans* extract through LC-MS/MS negative ion mode which shows Retention time (Rt) for each of the compounds. There is total 49 compounds shortlisted. The compounds were sorted by look on the Score Db and Score MFG that are closed to 100. From all the compounds with high scores obtained, the compounds with Diff (Db.ppm) and Diff (MFG.ppm) that has value within -2 to +2 are selected. All the 49 compounds categorized into their own organic class.

The metabolites were selected by referring at the Score Db and Score MFG values that are close to 100. From all the metabolites with high scores obtained, the metabolites with Diff (Db.ppm) and Diff (MFG.ppm) that has value within -2 to +2 was selected. The experimental data from the blank sample are used as a negative control to monitor the intensity of the background signal and carry-overs during GC–MS analyses. The metabolites found in the blank analysis were removed from the positive and negative ionisation analyses. The compounds identified was measured and determined by referring on chromatographic retention times, accurate monoisotopic mass values, natural abundance isotope patterns and higher energy collisional dissociation (HCD) fragmentation patterns. These four independent criteria for each compound with those of the authentic standards were matched to ensure accurate identification of compounds in the 50% EtOH-Aq *C. nutans* extraction. Some metabolites from different biological conditions were not included in the table. This is due to either low metabolite concentration below the instrument threshold for detection, metabolite degradation, poor fragmentation or

poor ionisation efficiency for a specific metabolite [58].

In both positive and negative ion mode **(Table 1 and 2)** LC-MS/MS analysis, sugars, sugar alcohols, amino acids, organics acids, and fatty acids were identified together with the metabolites. Most of the metabolites discovered in this study had previously been reported by other researchers. These are isovitexin, isoorientin, luteolin, quercetin, and riboflavin, which are commonly found in *C. nutans* extract [28]. Apart from the commonly found metabolites and reported earlier, few new metabolites were identified through this analysis such as pheophorbide A and pheophorbide B in positive mode analysis while alliin, threonic acid, and schizonepetoside E in negative mode analysis.

All the 73 differential metabolites were assigned to various chemical categories, including amino acids, carboxylic and their analogs, organic acids, alkaloids, flavonoids, phenolics, and others. From another classification perspective, these metabolites covered amides, phenols and alcohols, esters, sulfur compounds, and others. **Figure 4 and 5** shows the number of organic compounds present in both LC-MS/MS analysis. In positive ion mode, alpha amino acids present in high number which is 4 compounds while in negative ion mode is flavonoid present in high number which is 11 compounds. From the 73 compounds identified, 20.5% of the compounds which is 15 compounds are belonging to flavonoid organic class. From this, we can conclude that compounds present in 50% EtOH-Aq *C. nutans* extract mostly are flavonoids. These can be proven by that flavonoid is a polar compound where soluble in water as well as in organic solvents such as ethanol and methanol [3].

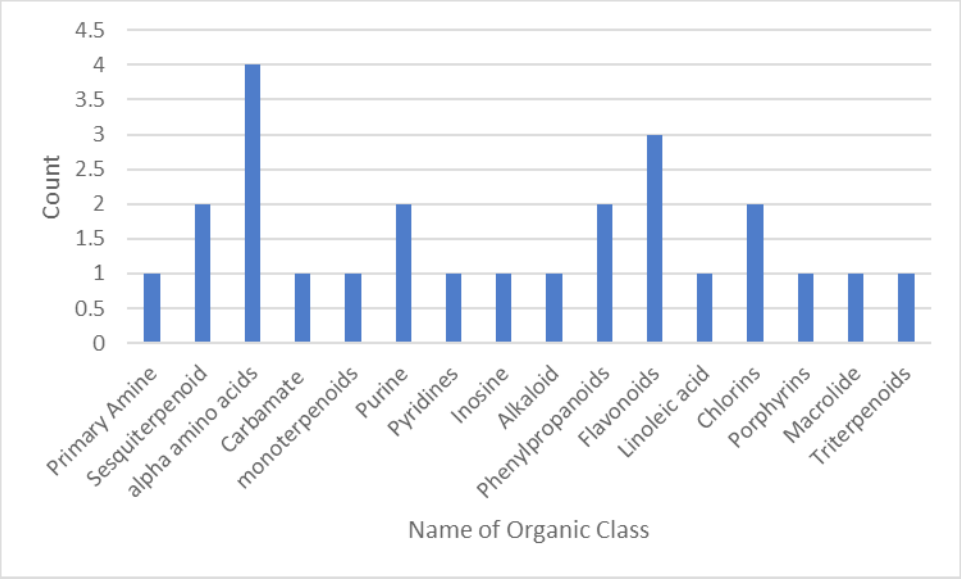


Figure 4: Number of compounds present in the positive ion mode LC-MS/MS analysis according to the organic class. 4 of 24 compounds belong to alpha amino acid while 3 compounds are belonging to flavonoid organic class. 2 compounds belonging for sesquiterpenoid, phenylpropanoids, purine and chlorins organics class, respectively.

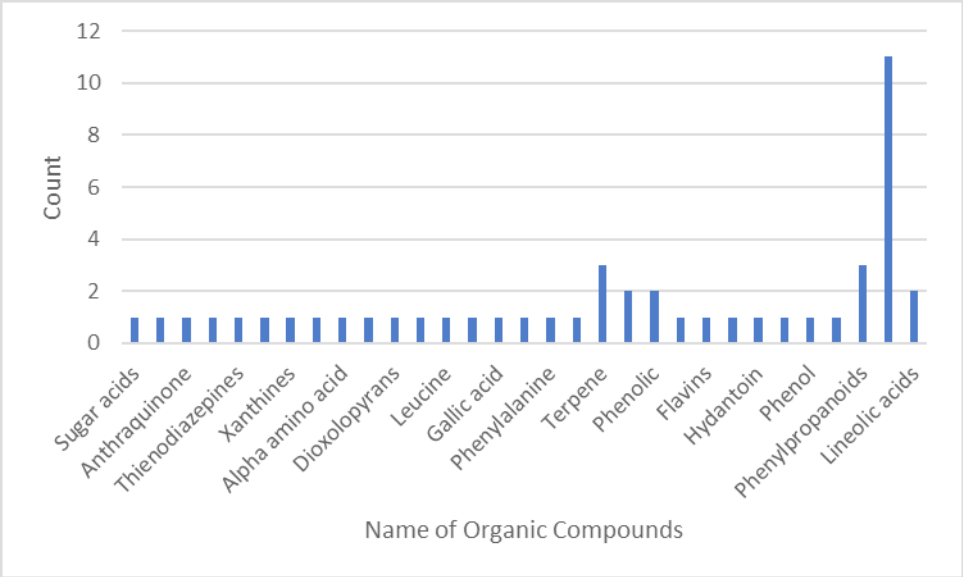


Figure 5: Number of compounds present in the negative ion mode LC-MS/MS analysis according to the organic class. Flavonoids organic class shows highest number of compounds that present in negative mode ion analysis which is 11 compounds from 49 compounds. Phenylpropanoids and terpene have 3 compounds, respectively.

A summary of the compounds and number of organic compounds that have been identified in the 50% EtOH-Aq *C. nutans* plant extract trough GC-MS analysis is provided in **Table 3** and **Figure 6**. The compounds were identified through mass spectrometry attached with GC. All the compounds were sorted down by referring at the percentage of quality where the only compounds have more than 80% of quality were selected. To date, the group of compounds that have been identified from *C. nutans* extraction through GC-MS analysis include alkaloids, terpenoids, triterpenoids, phenolics, sulfur containing glucosides and lipid related compounds [28]. There was few commonly identified and reported compounds in *C. nutans* leaves extraction such as lupeol, betulinic acid, succinate, coumaric acid, malic acid, and propanoic acid. Most of these commonly found compounds was identified in the GC-MS analysis but the compounds quality was not more than 80%. From the 20 metabolites, 5 metabolites are belonging to alpha amino acid group with more than 80% quality. All the 20 differential metabolites were assigned to various chemical categories, including amino acids, carboxylic, sugar acids and organic acids.

No	Rt	Area of peak	Name	Molecular formula	Mass	CAS	Quality	Class
1	5.1053	0.6893	L-Alanine	$C_3H_7NO_2$	89.0932	027844-07-1	87	Alpha amino acid
2	7.4342	0.271	L-Valine	$C_5H_{11}NO_2$	117.1463	007364-44-5	83	
3	9.1851	0.5966	L-Proline	$C_5H_9NO_2$	115.1305	007364-47-8	91	
4	11.205	0.2149	L-threonine	$C_4H_9NO_3$	119.1192	007537-02-2	83	
5	15.2046	1.124	L-Asparagine	$C_4H_8N_2O_3$	132.1179	055649-62-2	93	
6	10.1235	0.6054	Propanoic acid	$C_3H_6O_2$	74.0785	038191-87-6	95	Carboxylic acids
7	8.9334	1.2058	Glycerol	$C_3H_8O_3$	92.0938	006787-10-6	91	Sugar alcohols
8	13.757	0.2488	Trihydroxybutyric acid	$C_4H_8O_5$	136.1033	038191-88-7	81	
9	16.5321	0.3468	2-Keto-l-gluconic acid	$C_6H_{10}O_7$	194.1394	1000059-82-4	90	
10	9.5399	0.2868	Butanedioic acid	$C_4H_6O_4$	118.088	040309-57-7	91	Dicarboxylic acids

11	12.83	1.1226	Malic acid	$C_4H_6O_5$	134.0874	107241-82-7	94	Beta Hydroxy acids
12	13.3221	0.9462	Butanoic acid	$C_4H_8O_2$	88.1051	039508-23-1	83	Fatty Acyls
13	17.5392	0.7891	D-Galactose	$C_6H_{12}O_6$	180.1559	006736-94-3	93	Hexoses
14	17.8653	10.3996	beta.-D-Glucopyranose	$C_6H_{12}O_6$	180.1559	002775-90-8	94	
15	18.7809	10.7186	D-Glucose	$C_{18}H_{36}O_{18}$	540.4676	006736-97-6	95	Aldehydes
16	22.935	0.7325	D-Glucose	$C_{18}H_{36}O_{18}$	540.4676	055669-93-7	80	
17	21.7449	1.8394	alpha.-d-galactopyranoside	$C_8H_{16}O_6$	208.209	1000098-09-0	87	o-glycosyl
18	23.9364	0.3014	Maltose	$C_{12}H_{22}O_{11}$	342.2965	033428-94-3	87	
19	19.004	4.9117	Galactonic acid	$C_6H_{12}O_7$	196.1553	055400-16-3	93	Hydroxy acids
20	19.7994	2.6006	Myo-Inositol	$C_6H_{12}O_6$	180.1559	002582-79-8	87	Cyclohexanols

Table 3: List of organic compounds found through GC-MS analysis which shows retention time (Rt), area of the peak (%), chemical abstracts service number (CAS) and quality of compound detected (%) for each compound. There is total 20 compounds shortlisted based on the abundancy. All the 20 compounds categorized into their own organic class.

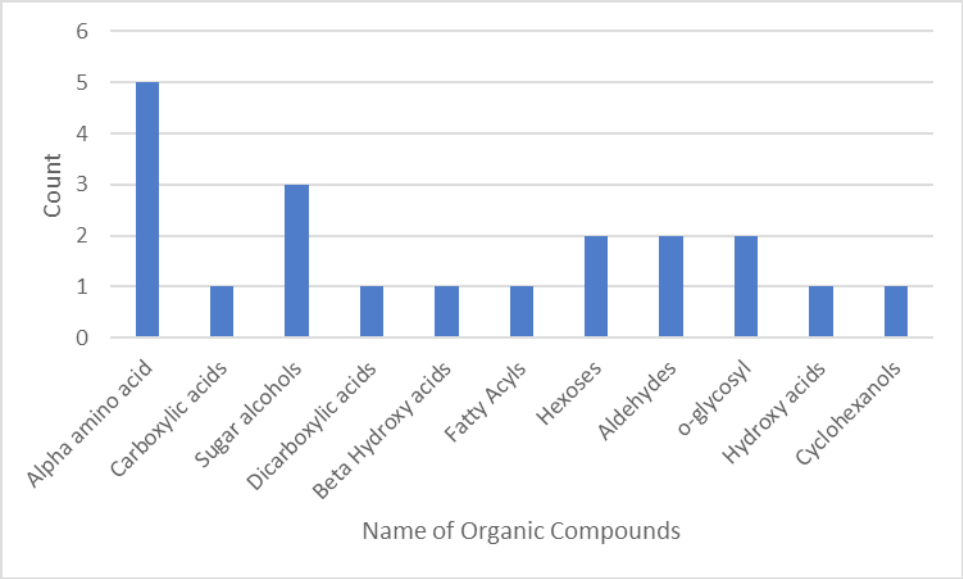
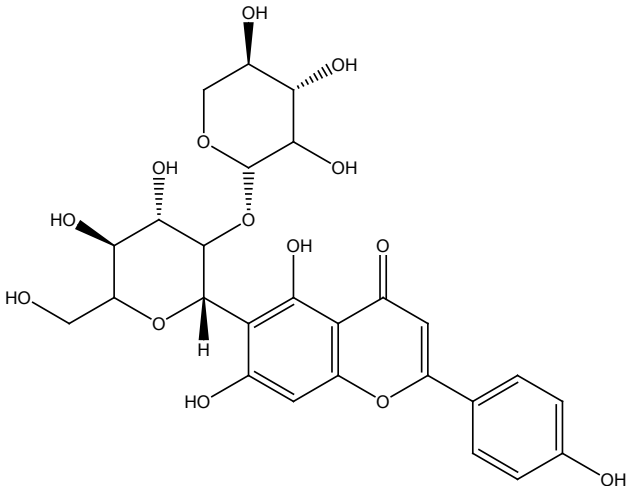
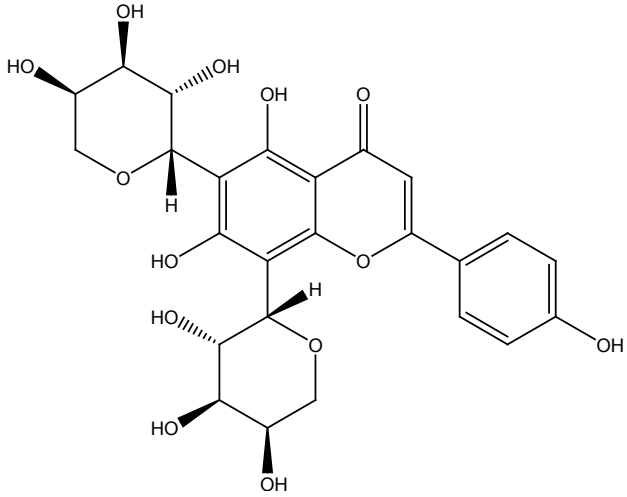
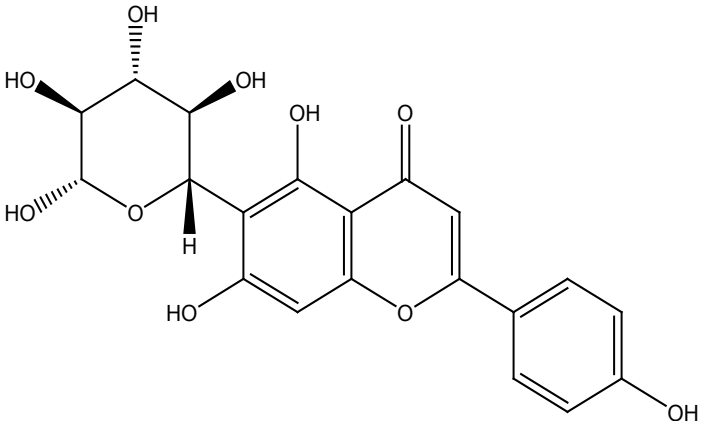


Figure 6: Number of compounds present in the GCMS analysis according to the organic class. From the 20 compounds, 5 compounds belong to alpha amino acid while 3 compounds belonging to sugar alcohols organic class.

Some metabolites are found in both positive and negative ion modes. The type of solvent, chromatography mode used, as well as the pH of buffers, can influence the response in a specific ionisation mode. **Table 4** shows that 5 metabolites together with their chemical structure and organic compound class that present in both ionisation mode. 3 of the same metabolites are belonging to flavonoid organic class while other 2 metabolites are belonging to alpha amino acid and inosine organic class. **Figure 7** is a Venn diagram showing the number of compounds present in negative ion mode, positive ion mode and compounds that present in both ion mode. There is a total of 25 compounds in positive ion mode and 49 compounds in negative ion mode. 5 compounds present in both ion mode which are isovitexin 2''-O-xyloside, 6,8-Di-C-beta-D-arabinopyranosyl apigenin, isovitexin5'-S-Methyl-5'-thioinosine, and alliin.

No	Name	Structure	Organic class
1	Isovitexin 2''-O-xyloside		Flavonoid
2	6,8-Di-C-beta-D-arabinopyranosyl apigenin		
3	Isovitexin		

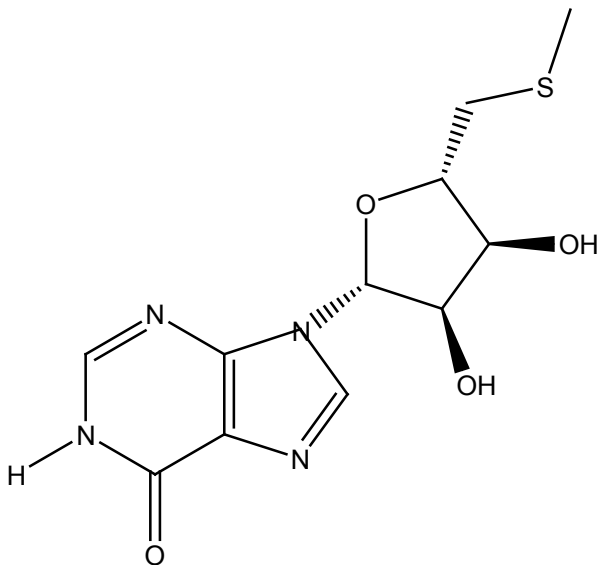
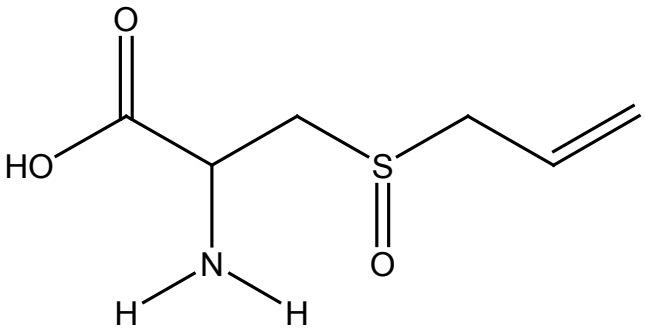
4	5'-S-Methyl-5'-thioinosine		Inosine
5	Alliin		Alpha amino acid

Table 4: List of compounds and the chemical structure which are found in both positive and negative mode ion of LC-MS/MS analysis. 5 compounds are found in both positive and negative ion mode which all the 5 compounds belong flavonoid, inosine and alpha amino acid organic class. 3 of the 5 compounds belonging to flavonoids organic class.

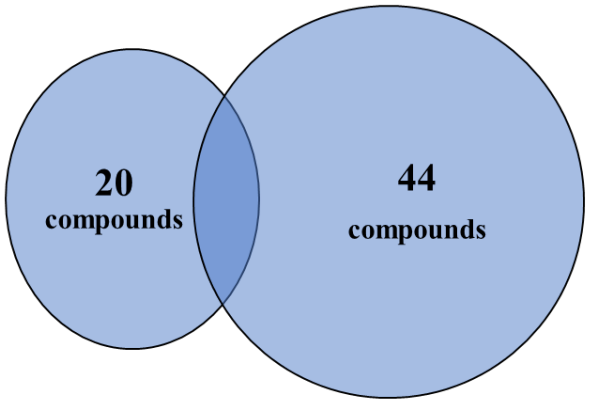


Figure 7: Venn diagram for number of compounds present in both positive and negative ion mode in LC-MS/MS analysis. There are 25 compounds in total, present in positive ion and 49 compounds present in negative ion analysis. From the total compounds, 5 compounds present in the positive ion and negative ion analysis.

Discussion

Extraction is the separation of compounds from plant tissues using selective solvents through standard procedures. During extraction, the role of solvents is to diffuse into solid plant material and solubilize compounds with similar polarity. Extraction efficiency is commonly affected by the chemical nature of phytochemicals, the method used for extraction, size of sample particle, the solvent used and also the presence of interfering substances between solvent and solute [48]. Apart from that, the yield of extraction depends on the polarity of solvent, pH, temperature used, extraction time, and composition of sample [61]. 50% EtOH-Aq was used in this study to extract the metabolites from *C. nutans* plants. The type of solvent was chosen based on the previous study carried out in the lab which identified the 50% EtOH- Aq extract of *C. nutans* as having potential immunomodulator effects in J774.2 macrophage cell line.

In this research, ultrasound assisted total extraction technique (sonication) was carried out to look over different types of compounds to be extracted from 50% EtOH-Aq of *C. nutans* leaves extraction. From this technique, little amount of extracts was obtained. However, it is more economical and convenient to be practiced compared to other extraction techniques such as microwave-assisted extraction [54]. Moreover, it minimizes the extraction temperature, amount of solvent used, and the extraction time. Other than that, all thermosensitive and unstable active compounds present in medicinal herb plants can be protected while extracting them using this technique since application of any extra heat is unnecessary during sonication [53]. Besides that, any desired solvent can be used in this technique to extract various type of natural compounds [54].

The dried leaves of *C. nutans* was grinded into fine powder before mixing with the solvents as this will help to rupture the plant material's organ, tissue, and cell structures. Thus, their active compounds will be accessible to the solvent used [49]. In addition, this will also increase the surface area for extraction by increasing the mass transfer of the soluble active compounds from the powdered leaves to the solvent used, which will increase the extraction rate [51]. This is because the soluble components in the solid material tend to diffuse into the solvent whenever a solid material and a solvent comes into contact with each other [49]. Drying method and type of solvent can influence the percentage yield of an extract. There is two method of drying method which is rotary evaporator and freeze-drying technique. In this study, freeze drying technique was used and this method was selected based on the previous study that conducted in our lab which is the study about drying technique of 50% EtOH-Aq extract of *C. nutans* (unpublished data). Based on that study, freeze drying technique yielded larger amount of extract, while rotary evaporator yielded fewer amount of extract which was in paste-like sticky texture. The percentage of yield of extract obtained rotary evaporator can only be estimated partially because of the extremely sticky paste nature of extract obtained from rotary evaporator could not be harvested completely as the extract adhered very firmly to the rotary flasks used.

From the ultrasound extraction techniques, small quantity of thick sticky extracts was obtained. After freeze drying technique, the 50% EtOH-Aq extracts were in golden brown color and the extracts appeared in crystal-like powder textures. Freeze-drying is a process where used to remove residual solvent from the extraction. This process is carried out to produce a dry powder that can be easily loaded into a cell. The main goal of freeze drying is to make the extract with good shelf stability that is unchanged after added with water again [36]. The solvent sublimates and is removed by vacuum, leaving a dry powder. During freeze-drying, the temperature is maintained sufficiently low that any remixing of the phase-separated polymer solution is prevented [19].

After the freeze drying, small quantity of crystal-like powder textures extracts was obtained which is 12.1% of yield. Sriwanthana et al. reported 15% yield of ethanol extract in the Soxhlet method [47]. It was indeed a much higher yield in comparison to the yield obtained in this experiment. The difference between the percentages of yield may possibly be due to the different methods used. As one of the advantages of Soxhlet extraction is the ability to produce large amount of extracts with much smaller quantity of solvent. However, this method is not suitable for thermolabile compounds as prolonged heating may lead to degradation of compounds [40]. Therefore, ultrasonication is feasible and useful for the extraction of thermosensitive and unstable compounds [53].

Metabolite identification is one of the most important aspects in metabolomics platform. In this platform, metabolites are rigorously identified by accurate mass, MS/MS fragmentation pattern, isotope pattern, and retention time to minimize the misidentification. Besides metabolite detection, the extraction and the separation of metabolites for the analysis are also important. Metabolite extraction is complicated because of the concentration range and physical diversity of metabolites that usually present in the plant [37]. Concentrating sample extracts and developing separate extractions specific for polar and non-polar metabolites are common strategies to overcome these challenges [14]. Many studies showed that extraction of metabolites of diverse polarity can be obtained efficiently using solvent mixtures [7][57].

Commonly solvents that will be used for the extraction are propane, supercritical CO_2, ethanol, water, methanol and butane. The extraction method using solvent work by dissolving the trichome heads in the liquid solvent which is separated from the plant material and then evaporated off in an oven or vacuum pump. The function of evaporation method will be able to extract pure compound and avoid solvent residuals [42]. Solvents fall into two group which is polar and non-polar. Non-polar solvents such as propane and butane extract the non-polar compounds like lipids, oils and waxes comprising the trichome heads. While polar solvents such as water and ethanol extract polar compounds. Water insoluble molecules, like chlorophyll a and chlorophyll b are naturally soluble in alcohols like ethanol [42].

Finding the potential biological active compounds in a plant extract is complicated as hundreds of active compounds might be present at various quantities and a single solvent is not capable to extract all compounds in a particular plant due to the diverse in polarity of the compounds [59]. The extraction and identification of metabolite from the plant material are generally affected by various factors inclding time, temperature, solvent concentration and solvent polarity. From this study, we found that there is a variation difference in the phytochemical's constituents compared to previous study for the *C. nutans* extraction by other researchers. Based on previous studies, most commonly metabolites that can found in *C. nutans* extraction are flavonoids, triterpenoids, steroids, phytosterols, phenolics and glycosides [28]. One of the reasons for variation caused in the metabolite identified is because of the different solvent and polarity usage for the extraction method [60]. Depending on chemical nature, various metabolites are extracted in solvents of different polarity as no single solvent may be reliable to extract all the metabolite compounds present in the plant material [26][31]. The solvents tend to diffuse into the solid plant materials during the extraction process and solubilize active compounds with the same polarity that have affinity towards the solvent [51]. In this research, the 1:1 ratio of solvent and mixture used (ethanol (ETOH) and water (Aq)) was chosen based on previous research conducted in lab (unpublished data). Based on that study, the percentage yield of 50% EtOH-Aq extract was greater than extracted with 100% ethanol. This proof the relationship between solvent polarity and the percentage of extraction yield where solvent with greater polarity produce larger extract yield. Based on Sarega et al, which had reported that the percentage yield of extraction increased with increasing polarity of solvents used [45]. The combined use of organic solvent which is ethanol (EtOH) and water (H_20) may facilitate the extraction of compounds that are soluble in water and ethanol. Both solvent is a polar solvent where it extracted most of the polar compounds from the pulverized *C. nutans* leaves. Based on the result obtained, polar compounds are easier to be extracted compared to non-polar compounds. Although both water and ethanol contain hydroxyl group that can form hydrogen bonding with the solute, water is more effective in extracting the solute because it has higher polarity and shorter chain [38]. These characteristics of water improved its capability to extract the polar compounds. This explains the significant observed between H_2O and EtOH.

Water could dissolve alkaloid and glycoside compounds, but ethanol was effective to extract sterol, flavonoid, phenolic, and alkaloid. There were less chemical compound of *C. nutans* leaves that had non polar properties. Previous research informed that methanol and ethanol can dissolve polar compounds, such as sugar, amino acid, glycoside compounds [23], phenolic compounds with low and medium molecular weights and medium polarity [33], aglycon flavonoid [12], anthocyanin, terpenoid, saponin, tannin, xantoxilin, totarol, quacinoid, lacton, flavone, phenone, and polyphenol [10]. Whereas water is effective to extract glycoside compounds, amino acid, and sugar [23], aglycon compounds [12] [34], vitamin C [11]. Ethyl acetate is effective to extract alkaloid, aglycon, and glycoside compound [23], sterol, terpenoid, and flavonoid [10].

Water is a low-cost, easy-to-obtain, nontoxic solvent with high polarity. It has the potential to extract polar metabolites such as inorganic salts, saccharides, amino acids, tannins, proteins, organic acid salts, alkaloid salts, and glycosides. Acid or alkaline water is used in certain extractions to increase the solubility of specific components. Through the formation of salts, acid water could extract alkaline materials such as alkaloids. Similarly, salt formation could be used to extract organic acids, anthraquinonoids, flavonoids, coumarinoids, phenols, and other acidic materials. Furthermore, the water would extract impurities such as proteins, pectins, mucilages, and inorganic salts, making target component extraction difficult [16].

On the other hand, ethanol has a high polarity and is water miscible. Ethanol is a type of hydrophilic organic solvent. Chemical constituents in plants can be extracted using ethanol in

various concentrations depending on their properties. Furthermore, ethanol is cheap, safe, and simple to concentrate. Aside from that, ethanol extract does not mould easily, and glycosides are difficult to hydrolyze in ethanol extract [16]. As a result, ethanol is one of the most commonly used solvents in laboratories and industrial production.

Apart from the solvent used for extraction, sample used for extraction also can be one of the factors to cause the variation in the metabolite identification in this research from previous research by other researchers. Environmental conditions, geographical location, cultivation practices, genetics, pre-harvesting and post-harvesting methods used, all of which are referred to as environmental factors, will cause variation in phytochemicals found in plants, even within the same species [17]. Plants can be influenced by a variety of factors, the most important of which are environmental factors, which force them to acclimate and change parts of their functioning in order to protect themselves. One of the primary causes of these changes is the presence of microorganisms, which cause changes in the physiology and development of plants [2]. When there is an interaction between a plant and a pathogen occurs, one of the changes that happen in the plant is the production of several kinds of compounds [25]. Primary and secondary metabolites play an important role in the plant's response to pathogen attacks. Many changes, including molecular and physiological changes, could occur during such interaction. The changes range from changes in primary metabolism, which can affect basic processes like photosynthesis, to modifications in the cell wall and in some plant organs, to the production of secondary metabolites, which can be toxic or trigger defensive signals in the plant [21].

The compounds extracted from the 50% EtOH-Aq *C. nutans* extraction can be volatile or non-volatile. To detect both type of compounds which is volatile and non-volatile present in the extraction, two analytical methods was used which is LC-MS/MS and GC-MS. Coupling chromatography to MS always gives an excellent solution to complex mixture analyses. Chromatographic separation of metabolites prior to MS analyses has several advantages which will be reduces matrix effects and ionisation suppression, separates isomers, provides additional and orthogonal data and allows for more accurate quantification of individual metabolites. This analysis is carried out using analytical techniques specialized in separation, identification and quantification. These techniques have high resolution, be very precise and very sensitive, and be able to analysis a wide variety of compounds of different chemical nature and origin [56].

Because the extraction may contain both volatile and nonvolatile compounds, GC-MS is appropriate for analyse both volatile and nonvolatile compounds after derivatization. GC is an excellent tool for complex metabolite mixture analyses because of the high resolution and reproducible chromatographic separations offered by the modern capillary [4]. Apart from that, the standardized MS electron ionisation energy of 70 eV gives a reproducible mass spectra and highly transferable electron ionisation MS spectral libraries where it allows the compound identification through mass spectral library matching [29]. GC requires volatile and thermally stable analytes [5]. However, relatively few compounds meet this requirement in their native state such as short chain alcohols, acid, esters, and hydrocarbons [6]. Many other compounds can be analysed by GC after derivatization. Although derivatization is necessary for certain GC analyses, derivatization can introduce variability and produce artifacts [30]. Most of the time, GC-MS is only detecting volatile, thermally stable, and energetically stable compounds where it is less amenable to large highly polar metabolites due to their poor volatility. To detect the large highly polar metabolites due to their poor volatility, another type of chromatographic techniques such as LC-MS was used.

LC-MS/MS is an important tool in metabolite profiling and can be used for targeted or non-targeted metabolites identification. LC-MS/MS utilizing two columns of different selectivity gives an effective platform for separating both polar and non-polar compounds simultaneously [18]. Non-volatile compounds, vitamins, amino acids, protein, and peptides are detected in the extraction using LC-MS/MS. It is very useful for studying purity and impurity profiles in drugs. The ionisation technique used in LC-MS analyses can also have a significant impact on metabolite profiles. There are two ionisation techniques: electrospray ionisation (ESI) and atmospheric pressure chemical ionisation (APCI). In this research LC-MS/MS with ESI ionisation was used. ESI is commonly used to detect semi-polar and polar compounds, whereas APCI is more suitable to detect neutral or less polar compounds.

Apart from using ESI ionisation technique, both positive and negative ionisation LC-MS/MS analyses was carried out in this research. Based on Tolonen (2004), Many modern MS instruments have capability of fast polarity switching during data acquisition and have been exploited in simultaneous acquisition of both positive and negative ion mode data [52]. The use of both positive and negative ionisation in LC-MS analyses gives more comprehensive metabolome coverage than the use of a single polarity [15]. In this research, several metabolites such as iridoid, indoles and terpenes were detected only in the negative ion mode, whereas others were observed only in the positive ion mode. Most of the compounds in LC-MS/MS analyses was flavonoids and most of the flavonoid compounds are detected in negative ion mode. Similarly, Nordstrom et al. (2008) said that more than 90% of the human blood plasma ions observed in the positive ion ESI mode were not found in the negative ion mode and vice versa [35].

Conclusion

Metabolite profiling of the 50% EtOH-Aq *C. nutans* extraction have suggested that *C. nutans* is a rich source of flavonoids, phenolics, and terpenoids, triterpenoids, and sugar compounds. However, their retention in the extract id largely influenced by the plant preparation and extraction method. The identified compounds occupy many biological properties. GC-MS and LC-MS/MS analysis of phytoconstituents in plants gives a clear picture of the pharmaceutical value of the plant. Thus, GC-MS and LC-MS/MS analysis is the first step towards understanding the nature of medicinal properties in this medicinal plant and this metabolite profiling study will be helpful for further detailed study on the plant's metabolite. The mechanism of action of the identified compounds in relation to a specific treatment should be explained. More experimental studies are also likely to be conducted to substantiate and describe the relationship between the isolated phytochemicals from *C. nutans* and their corresponding pharmacological effects.

Acknowledgement

This work is supported and funded by Universiti Sains Malaysia Research University (RUI) Grant 1001.CIPPT.8012204. All authors report no conflicts of interest relevant to this article.

The authors confirm contribution to the paper as follows:

Ravi Kumar Kalaichelvam: monitored data collection, analysed the data, drafted, and revised the paper. Lim Vuanghao and Ida Shazrina Ismail: designed the experiments, reviewed, and revised the manuscript. All authors reviewed the results and approved the final version of the manuscript.

References

1. Agostini-Costa TS, Vieira RF, Bizzo HR, Silveira D, Gimenes MA (2012) Secondary Metabolites. Chromatography and Its Applications.
2. Allwood JW, Cheung W, Xu Y, Mumm R, Vos RCH, et al. (2014) Metabolomics in melon: A new opportunity for aroma analysis. Phytochemistry 99:61-72.
3. Awouafack MD, Tane P, Morita H (2017) Isolation and Structure Characterization of Flavonoids. Flavonoids - From Biosynthesis to Human Health.
4. Babushok VI, Linstrom PJ, Reed JJ, Zenkevich IG, Brown RL, et al. (2007) Development of a database of gas chromatographic retention properties of organic compounds. J Chromatogr A 1157:414-421.
5. Begley P, Francis-McIntyre S, Dunn WB, Broadhurst DI, Halsall A, et al. (2009) Development and performance of a gas chromatography-time-of-flight mass spectrometry analysis for large-scale nontargeted metabolomic studies of human serum. Anal Chem 81:7038-7046.
6. Broeckling CD, Huhman DV, Farag MA, Smith JT, May GD, et al. (2005) Metabolic profiling of Medicago truncatula cell cultures reveals the effects of biotic and abiotic elicitors on metabolism. J Exp Bot 56:323-336.
7. Bruce SJ, Tavazzi I, Parisod V, Rezzi S, Kochhar S, Eet al. (2009) Investigation of human blood plasma sample preparation for performing metabolomics using ultrahigh performance liquid chromatography/ mass spectrometry. Anal Chem 81:3285-3296.
8. Chiu HI, Che Mood CNA, Mohamad Zain NN, Ramachandran MR, Yahaya N, et al. (2021) Biogenic Silver Nanoparticles of *Clinacanthus nutans* as Antioxidant with Antimicrobial and Cytotoxic Effects. Bioinorg Chem Appl 2021:9920890.
9. Chong HW, Rezaei K, Chew BL, Lim V (2018) Chemometric Profiling of Clinacanthus nutans Leaves Possessing Antioxidant Activities using Ultraviolet-visible Spectrophotometry. Chiang Mai J Sci 45:1519-1530.
10. Cowan MM (1999) Plant products as antimicrobial agents. Clin Microbiol Rev 12:564-582.
11. Dalimartha S (2003) Atlas Tumbuhan Obat Indonesia Jilid 3 / Setiawan Dalimartha (edisi 1 ; jilid 4). PUSTAKA PEMBANGUNAN.
12. Dehkharghanian M, Adenier H, Vijayalakshmi MA (2010) Study of flavonoids in aqueous spinach extract using positive electrospray ionisation tandem quadrupole mass spectrometry. Food Chem 121:863-870.
13. Devasvaran K, Hanim Baharom N, Wen Chong H, Nuraika Ramli R, Ing Chiu H, et al. (2020) Quality assessment of *Clinacanthus nutans* leaf extracts by GC-MS-based metabolomics. Current Science 119:641-648.
14. Everroad RC, Yoshida S, Tsuboi Y, Date Y, Kikuchi J (2012) Concentration of metabolites from low-density planktonic communities for environmental metabolomics using nuclear magnetic resonance spectroscopy. J Vis Exp e3163.
15. Farag MA, Huhman DV, Lei Z, & Sumner LW (2007) Metabolic profiling and systematic identification of flavonoids and isoflavonoids in roots and cell suspension cultures of Medicago truncatula using HPLC–UV–ESI–MS. Phytochemistry 68:342-354.
16. Feng W, Li M, Hao Z, Zhang J (2019) Analytical Methods of Isolation and Identification. Phytochemicals in Human Health.
17. Fong SY (2015) Genetic, phytochemical and bioactivity studies of Clinacanthus nutans (Burm. f.) Lindau (Acanthaceae). RMIT University.
18. François I, Villiers A, Tienpont B, David F, Sandra P (2008) Comprehensive two-dimensional liquid chromatography applying two parallel columns in the second dimension. J Chromatogr A 1178:33-42.
19. Ghalia MA, Dahman Y (2016) Advanced nanobiomaterials in tissue engineering: Synthesis, properties, and applications. Nanobiomaterials in Soft Tissue Engineering. William Andrew Publishing 5;141-172.
20. Graikou K, Kapeta S, Aligiannis N, Sotiroudis G, Chondrogianni N, et al. (2011) Chemical analysis of Greek pollen-Antioxidant, antimicrobial and proteasome activation properties. Chem Cent J 5:33.
21. Heuberger AL, Robison FM, Lyons SMA, Broeckling CD, Prenni JE (2014) Evaluating plant immunity using mass spectrometry-based metabolomics workflows. Front Plant Sci.
22. Ooi SH, Mohamed NMN, Kalaichelvam RK, Lim V, Ismail IS (2021) Effects of Clinacanthus nutans Extracts on Cytokine Secretion in PMA-Induced U937 Macrophage Cells. Research Journal of Pharmacognosy 8:27-35.

23. Houghton PJ, Raman A (2012) Laboratory handbook for the fractionation of natural extracts.

24. Huang D, Guo W, Gao J, Chen J, Olatunji J (2015) Clinacanthus nutans (Burm. f.) Lindau ethanol extract inhibits hepatoma in mice through upregulation of the immune response. Molecules 20:17405-17428.

25. Ibarra-Estrada E, Soto-Hernández RM, Palma-Tenango M (2016) Metabolomics as a Tool in Agriculture. Metabolomics: Fundamentals and Applications 148-168.

26. Iloki-Assanga SB, Lewis-Luján LM, Lara-Espinoza CL, Gil-Salido AA, Fernandez-Angulo D, et al. (2015) Solvent effects on phytochemical constituent profiles and antioxidant activities, using four different extraction formulations for analysis of Bucida buceras L. and Phoradendron californicum Complementary and Alternative Medicine. BMC Res Notes 8:396.

27. Kanani H, Chrysanthopoulos PK, Klapa MI (2008) Standardizing gc–ms metabolomics. J Chromatogr B Analyt Technol Biomed Life Sci 871:191-201.

28. Khoo LW, Kow SA, Lee MT, Tan CP, Shaari K, et al. (2018) A comprehensive review on Phytochemistry and pharmacological activities of *Clinacanthus nutans* (Burm. F.) Lindau. Evid Based Complement Alternat Med 2018:9276260.

29. Kind T, Wohlgemuth G, Lee DY, Lu Y, Palazoglu M, et al. (2009) FiehnLib: Mass spectral and retention index libraries for metabolomics based on quadrupole and time-of-flight gas chromatography/mass spectrometry. Anal Chem 81:10038-10048.

30. Koek MM, Bakels F, Engel W, Van Den Maagdenberg A, Ferrari MD, et al. (2010) Metabolic profiling of ultrasmall sample volumes with GC/MS: From microliter to nanoliter samples. Anal Chem 82:156-162.

31. Lapornik B, Prošek M, Wondra A (2005) Comparison of extracts prepared from plant by-products using different solvents and extraction time. Journal of Food Engineering 71:214-222.

32. Liigand P, Kaupmees K, Haav K, Liigand J, Leito I, et al. (2017) Think Negative: Finding the Best Electrospray Ionization/MS Mode for Your Analyte. Anal Chem 89:5665–5668.

33. Lin HY, Kuo YH, Lin YL, Chiang W (2009) Antioxidative Effect and Active Components from Leaves of Lotus (Nelumbo nucifera). J Agric Food Chem 57:6623-6629.

34. Liu J, Wang C, Wang Z, Zhang C, Lu S, et al. (2011) The antioxidant and free-radical scavenging activities of extract and fractions from corn silk (Zea mays L.) and related flavone glycosides. Food Chemistry 126:261-269.

35. Nordström A, Want E, Northen T, Lehtiö J, Siuzdak G (2008) Multiple ionization mass spectrometry strategy used to reveal the complexity of metabolomics. Anal Chem 80:421-429.

36. Oetjen, G, Haseley P (2004) Freeze-drying.

37. Patti GJ (2011) Separation strategies for untargeted metabolomics. J Sep Sci 34:3460-3469.

38. Pin KY, Chuah AL, Rashih AA, Mazura M, Fadzureena J, et al. (2010) Antioxidant and anti-inflammatory activities of extracts of betel leaves (Piper betle) from solvents with different polarities. Journal of Tropical Forest Science 22:448-455.

39. Putwatana P, Sanmanowong P, Oonprasertpong L, Junda T, Pitiporn S, et al. (2009) Relief of radiation-induced oral mucositis in head and neck cancer. Cancer Nurs 32:82-87.

40. Raks V, Al-Suod H, Buszewski B (2018) Isolation, Separation, and Preconcentration of Biologically Active Compounds from Plant Matrices by Extraction Techniques. Chromatographia 81:189-202.

41. Revathi D, Rajeswari M (2015) Phytochemical analysis of *Guettarda speciosa* Linn. Asian J Plant Sci Res 5:1-4.

42. Ritchie H (2018) Solvent polarity in Cannabis extraction. Terpenes and Testing Magazine.

43. Rohloff J (2015) Analysis of polyphenols and cyclic compounds in plants using derivatization techniques in combination with GC-MS-based metabolite profiling. Molecules 20:3431-3462.

44. Sahri Z, Nordin S, Harun H (2012) Malaysia indigenous herbs knowledge representation.

45. Sarega N, Imam M, Ooi D, Chan K (2016) Phenolic rich extract from Clinacanthus nutans attenuates hyperlipidemia-associated oxidative stress in rats. Oxid Med Cell Longev 2016:4137908.

46. Sakdarat S, Shuyprom A, Ayudhya TD, Waterman PG, Karagianis G (2008) Chemical composition investigation of the Clinacanthus nutans (Burm. F.) lindau leaves. Suranaree University of Technology.

47. Sriwanthana B, Chavalittumrong P (1996) Effect of Clinacanthus nutans on human cell-mediated immune response in vitro. Thai Journal Pharmacy Science 20:261-267.

48. Stalikas CD (2007) Extraction, separation, and detection methods for phenolic acids and flavonoids. J Sep Sci 30:3268-3295.

49. Sukhdev SH, Suman PSK, Gennaro L, Dev DR (2008) Extraction technologies for medicinal and aromatic plants. International Centre For Science and High Technology Trieste.

50. Teshima K, Kaneko T, Ohtani K, Kasai R (1997) C-glycosyl flavones from Clinacanthus nutans. Natural Medicines 51:557.

51. Tiwari P, Kumar B, Kaur M, Kaur G, Kaur H (2011) Phytochemical Screening and Extraction A Review. Internationale Pharmaceutica Sciencia 1:98-106.

52. Tolonen A, Uusitalo J (2004) Fast screening method for the analysis of total flavonoid content in plants and foodstuffs by high-performance liquid chromatography/electrospray ionization time-of-flight mass spectrometry with polarity switching. Rapid Commun Mass Spectrom 18:3113-3122.

53. Wang J, Zhao YM, Tian YT, Yan CL, Guo CY (2013) Ultrasound-assisted extraction of total phenolic compounds from inula helenium. The Scientific World Journal 2013:157527.

54. Wang L, Weller CL (2006) Recent advances in extraction of nutraceuticals from plants. Trends in Food Science and Technology 17:300-312.

55. WHO Policy Perspectives on Medicines-Traditional Medicine-Growing Needs and Potential WHO Policy Perspectives on Medicines Traditional Medicine-Growing Needs and Potential (2002).

56. Wishart DS (2008) Metabolomics: applications to food science and nutrition research. Trends in Food Science & Technology 19:482-493.

57. Yanes O, Tautenhahn R, Patti GJ, Siuzdak G (2011) Expanding Coverage of the Metabolome for Global Metabolite Profiling. Anal Chem 83:2152-2161.

58. Yuan M, Breitkopf SB, Yang X, Asara JM (2012) A positive/negative ion–switching, targeted mass spectrometry–based metabolomics platform for bodily fluids, cells, and fresh and fixed tissue. Nat Protoc 7:872-881.

59. Yuliana ND, Khatib A, Verpoorte R, Choi YH (2011) Comprehensive extraction method integrated with NMR metabolomics: A new bioactivity screening method for plants, adenosine a1 receptor binding compounds in orthosiphon stamineus benth. Anal Chem 83:6902-6906.

60. Yusuf SNAM, Mood CNAC, Ahmad NH, Sandai D, Lee CK, et al. (2020) Optimization of biogenic synthesis of silver nanoparticles from flavonoid-rich Clinacanthus nutans leaf and stem aqueous extracts. Royal Society Open Science 7:200065.

61. Zieliński H, Kozłowska H (2000) Antioxidant activity and total phenolics in selected cereal grains and their different morphological fractions. J Agric Food Chem 48:2008-2016.