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OPTIMISATION OF THE EXTRACTION METHOD OF RED *Christia vespertilionis* LEAVES TO YIELD BIOACTIVE PHTYOCHEMICALS AS MONITORED BY GAS CHROMATOGRAPHY-MASS SPECTROMETRY

(Pengoptimuman Kaedah Pengekstrakan Pada Daun Merah *Christia vespertilionis* untuk Menentukan Fotokimia Bioaktif Melalui Kromatografi Gas-Spektrometri Jisim)

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Abstract

Christia vespertilionis (L. f.) Bakh. f. is well-known for treating various contagious diseases. This plant has been recognised among researchers and locals to have anti-inflammatory properties and has thus become popular for treating cancer. Two types of *C. vespertilionis* are acknowledged which are green and red. The green *C. vespertilionis* has been widely studied by many researchers, however just a few have studied the red type. This study was carried out to optimise the extraction method of red *C. vespertilionis* leaves by different extraction techniques (maceration and Soxhlet extraction) and solvents (methanol and ethanol) to yield bioactive phytochemicals using gas chromatography-mass spectrometry (GC-MS). The components were identified through GC-MS via comparisons as guided by the National Institute of Standards and Technology Mass Spectral Library 2011 (NIST 11, version 2.0g). According to the four (4) samples of red *C. vespertilionis* leaves using maceration of methanol (RMM), maceration of ethanol (RME), Soxhlet extraction of methanol (RSM), and Soxhlet extraction of ethanol (RSE), seventy-one (71) bioactive phytochemicals were identified. Eleven (11) major bioactive phytochemicals (abundance of > 4%) were identified namely, acetic acid, butyl ester; 1-butanol, 3-methyl-, acetate; heptanoic acid, propyl ester; hexanoic acid, 3-oxo-, ethyl ester; phenol, 3,5-bis(1,1-dimethylethyl)-; 1-octadecene; 4-O-methylmannose; .alpha.-d-mannofuranoside, methyl; 2-undecene, 9-methyl-, (E)-; n-hexadecanoic acid; and 1-octadecanol. Only seven (7) out of eleven (11) compounds were reported to have biological activities. Among those samples, RSM was the most effective using correlation coefficient between abundance (%) and retention time (minute(s)) with a significant difference at $P < 0.05$.

Keywords: *Christia vespertilionis*, gas chromatography-mass spectrometry, phytochemicals, maceration, Soxhlet extraction

Abstrak

Christia vespertilionis (L. f.) Bakh. f. dikenali secara meluas dalam merawat pelbagai penyakit berjangkit. Tumbuhan ini diakui di kalangan penyelidik dan penduduk tempatan tentang kelebihannya sebagai salah satu ubat tradisional yang mempunyai sifat

anti-radang serta digunakan secara meluas dalam merawat barah. Terdapat dua jenis *C. vespertilionis* iaitu jenis hijau dan merah. *C. vespertilionis* hijau telah banyak dikaji oleh penyelidik dan hanya sedikit yang dikaji untuk jenis merah. Kajian ini dijalankan untuk mengoptimumkan kaedah pengekstrakan daun *C. vespertilionis* merah dengan teknik pengekstrakan yang berbeza (pengekstrakan maserasi dan Soxhlet) dan pelarut (metanol dan etanol) untuk menentukan fitokimia bioaktif melalui kromatografi gas-spektrometri jisim (GC-MS). Komponen tersebut dikenal pasti melalui GC-MS dengan membuat perbandingan dari data rujukan Institut Piawaian dan Teknologi Nasional 2011 (NIST11-MS, version 2.0g). Berdasarkan 4 sampel daun merah *C. vespertilionis* melalui maserasi metanol (RMM), maserasi etanol (RME), pengekstrakan Soxhlet methanol (RSM) dan pengekstrakan Soxhlet etanol (RSE), tujuh puluh satu (71) sebatian fitokimia dikenal pasti. Sebelas (11) sebatian fitokimia utama (> 4 % kawasan puncak) juga dikenalpasti seperti asid asetik, butil ester; 1-butanol, 3-metil-, asetat; asid heptanoik, propil ester; asid heksanoik, 3-okso-, etil ester; fenol, 3,5-bis (1,1-dimetiletil)-; 1-oktadekena; 4-O-metilmanos; .alpha.-d-manofurosida, metil; 2-undekena, 9-metil-, (E)-; asid n-heksadekanoik dan 1-oktadekanol. Hanya tujuh (7) daripada sebelas (11) sebatian yang dilaporkan mempunyai aktiviti biologi. Di antara sampel tersebut, RSM adalah yang paling berkesan dengan menggunakan pekali korelasi antara kebanyakan (%) dan masa pengekalan (min) dengan perbezaan yang signifikan pada $P < 0.05$.

Kata kunci: *Christia vespertilionis*, kromatografi gas-spektrometri jisim, fitokimia, maserasi, pengekstrakan Soxhlet

Introduction

Christia vespertilionis (L. f.) Bakh. f. is a family of Fabaceae that is known as butterfly wing because of its shape, which is similar to that of a butterfly wing. This plant possesses the ability to cure several diseases due to it having various biological activities such as anti-cancer, anti-inflammatory, anti-proliferative and anti-plasmodial properties [1, 2, 3]. In Malaysia, *C. vespertilionis* leaves are consumed by cancer patients and have gained great popularity among Malaysians, including researchers who are keen to explore and discover the real potential of this plant.

C. vespertilionis is known to potentially exhibit various bioactive secondary compounds that can be used in food and pharmaceutical areas [4]. Previous studies revealed the presence of bioactive secondary compounds in *C. vespertilionis* such as alkaloid, phenol, fatty acid, triterpene and alcohol [2]. The aerial part of *C. vespertilionis* is revealed to contain corynoxidine and palmitine [2]. *C. vespertilionis* also possesses great anti-proliferative activities in MTC cells and is excellent in anti-plasmodial activities using aqueous-methanol extracts [1,2]. Other studies showed that *C. vespertilionis* extract has a high inhibition on cancer cells such as human medullary thyroid carcinoma and human intestinal neuroendocrine tumours [5]. In addition, *C. vespertilionis* exhibits moderately as an anti-malaria which is against the 3D7 malaria parasite and literally can be used for malaria treatment [6].

Today, people have developed high interest in traditional plants, as a substitute for synthetic drugs. Plant comes from nature and safe to use. It is also low cost and has less negative side effects [7]. However, the World Health Organization (WHO) has noted that inappropriate practices of using traditional plants for treatment can have dangerous effects. Therefore, further study is necessary to ascertain the efficacy and safety of plants for human consumption. While many studies of *C. vespertilionis* have been conducted, there is no study available on bioactive phytochemicals of red *C. vespertilionis* leaves using gas chromatography-mass spectrometry (GC-MS). GC-MS is one of the techniques that separates individual compounds in the form of a mass spectrum and measurement of the abundance of chromatography [8]. Identification of compounds in GC-MS is achieved by comparing mass spectrum of sample with the reference National Institute of Standards and Technology Mass Spectral Library 2011 (NIST11-MS) based on matching system.

Prior to that, before GC-MS, red *C. vespertilionis* leaves need to undergo extraction to separate the target products from the raw materials. Currently, solvent extraction is widely used in natural plants and herb medicines. Selection of solvent is also a crucial part for solvent extraction. The solvent with a shorter differences of polarity value to the polarity of the solute, is expected to perform better. As regarding the technique of extraction, there are two commonly used methods namely as maceration and Soxhlet extraction.

These methods were most of the time given acceptable results, however Soxhlet can be time-consuming although requires a small amount of sample compared to maceration. Therefore, this research aims to optimise the extraction method of red *C. vespertilionis* leaves in different extraction techniques (maceration and Soxhlet extraction) and solvents (methanol and ethanol) to yield bioactive phytochemicals using GC-MS.

Materials and Methods

Plant material

Red *C. vespertilionis* was collected from Floranika Nursery Sungai Buloh, Selangor (Malaysia), located at a latitude and longitude of 3° 13' 6.7764" N, 101° 34' 18.1704" E, respectively. The voucher specimen was authenticated by Dr Yong Kien Thai from Plant Taxonomy, Rimba Ilmu, University of Malaya. The voucher specimen of red *C. vespertilionis* (KLU 50025) was placed at the herbarium of the University of Malaya, and left to air-dry for 7 to 8 days. Leaves were ground with a blender to obtain coarse powder for extraction and were kept in a closed jar until further use.

Maceration

1 g of dried leaves powder of red *C. vespertilionis* was immersed into a 100 ml of methanol, MeOH (Merck, Germany), and absolute ethanol, EtOH (VWR Chemicals, American) in a 250 ml conical flask, separately. The samples were left in a water bath at 40 °C for 48 hours. The extracts were filtered and evaporated with rotary evaporator at 45 °C until they were concentrated. The extracts were filtered again to remove any solid particles and kept tightly closed in a microcentrifuge tube at 4 °C until further use.

Soxhlet extraction

1 g of dried leaves powder of red *C. vespertilionis* was extracted with 200 ml of MeOH (Merck, Germany), and absolute EtOH (VWR Chemicals, American) using a Soxhlet apparatus, separately. The samples were left in a water bath at 40 °C for 48 hours. The extracts were filtered and evaporated using a rotary evaporator at 45 °C until they were concentrated. The extracts were filtered again to remove any solid particles and kept tightly closed in a microcentrifuge tube at 4 °C until further use.

Gas chromatography-mass spectrometry analysis

Samples were diluted to 500 ppm with the respective solvents into 1.5 ml vials. The GC-MS analysis was done at the IPPP Central Laboratory Facilities, University Malaya, Kuala Lumpur, Malaysia. Model of GCMS-QP2010 Ultra (Shimadzu, Tokyo, Japan) was used for the GC-MS analysis. This model is a single quadrupole gas chromatograph-mass spectrometer that produces stable and affordable analyses for complex compounds. 0.5 µl of the sample was auto-injected into the system. The system was supplied with a capillary column of RTX5MS with length x diameter of 30.0 m × 0.25 mm and a 0.25 µm of thickness. The injection temperature was set to 200 °C which possessed a splitless injection mode. The initial temperature was 50 °C (3 minutes) with an accelerating rate of 10 °C (1 minute) to 300 °C (10 minutes). Helium gas was used with 47.8 cm/second of linear velocity. Electron ionisation (EI) mode at 70 eV with a spectral range of 35 m/z-500 m/z was performed for mass spectra results. The ion source temperature was fixed at 150 °C and the interface temperature was at 230 °C with a solvent cut-off time of 3 minutes. The start time was set at 3.0 minutes and the final time was adjusted at 33 minutes. The total flow programmed was 21.6 ml/minute with a column flow of 1.69 ml/minute. The compounds were determined based on the interpretation of mass spectrum with standard reference spectral using the National Institute of Standards and Technology Mass Spectral Library 2011 (NIST11) version 2.0g databases.

Statistical analysis

The correlation between abundance and retention time of the sample was determined using GraphPad Prism 8.0.2, where Pearson's correlation coefficient test was conducted and the difference was considered statistically significant when $P < 0.05$. Chemical structure drawing was applied on the website: https://chemwriter.com/.

Results and Discussion

GC-MS chromatogram and bioactive phytochemicals

GC-MS is important to identify bioactive phytochemicals in a complex compound using a technique of separation in a GC system and measuring on a mass spectrometer that comes with an electron ionisation (EI) ion source. There are two electron ionisation (EI) main spectrum library: main and replicate with a total of 212,961 and 30,932 mass spectra, respectively. NIST11-MS library has a total of 346,757 retention index for 70,835 compounds that were extracted based on column type, column class, data type and program type [9]. Through GC-MS analysis showed seventy-one (71) phytochemicals from four (4) samples that consist of major and minor compounds. Major compounds consisted of an abundance larger than 4% [10] as shown in Table 3. The conditions of the samples were shown in Table 1.

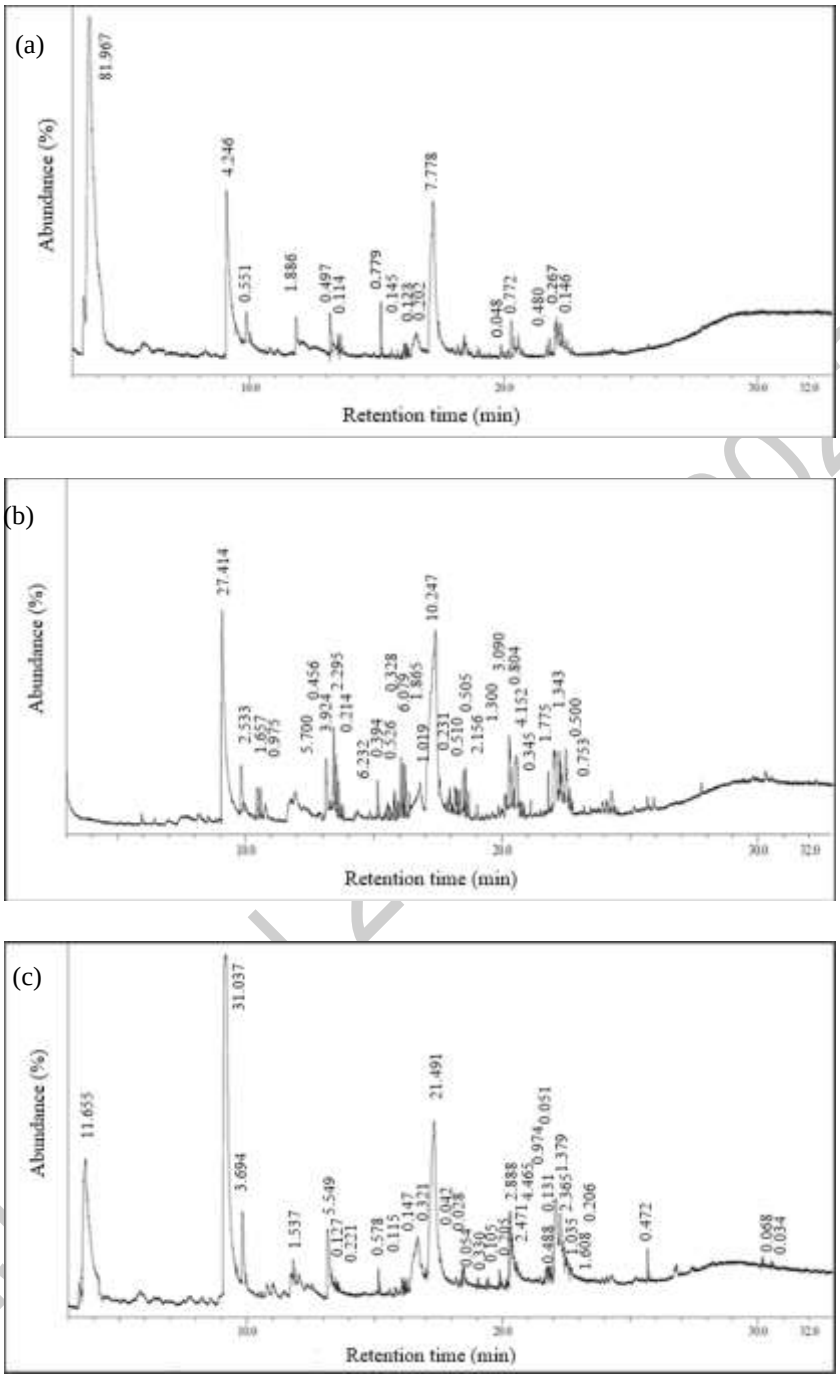
Bioactive phytochemicals of the samples were presented in Table 2 and the GC-MS chromatogram of the abundance of samples shown in Figure 1. GC-MS analysis for RMM resulted in a total of sixteen (16) peaks (according to Figure 1(a) and Table 2). There are three (3) major compounds, namely: acetic acid, butyl ester (81.967%); 1-butanol, 3-methyl-, acetate (4.246%) and 4-O-methylmannose (7.778%). RME resulted in a total of thirty (30) peaks (according to Figure 1(b) and Table 2). There were seven (7) major compounds, namely: 1-butanol, 3-methyl-, acetate (27.414%); heptanoic acid, propyl ester (5.700%); phenol, 3,5-bis(1,1-dimethylethyl)- (6.232%); 1-octadecene (6.079%); .alpha.-d-mannofuranoside, methyl (10.247%); 2-undecene, 9-methyl-, (E)- (5.505%) and 1-octadecanol (4.152%).

Furthermore, RSM resulted in a total of thirty-three (33) peaks (according to Figure 1(c) and Table 2). Five (5) major compounds were identified, namely: acetic acid, butyl ester (11.655%); 1-butanol, 3-methyl-, acetate (31.037%); hexanoic acid, 3-oxo-, ethyl ester (5.549%); 4-O-methylmannose (21.491%) and n-hexadecanoic acid (4.465%). Lastly, RSE resulted in a total of thirty-seven (37) peaks (according to Figure 1(d) and Table 2). Four (4) major compounds comprised: 1-butanol, 3-methyl-, acetate (6.414%); phenol, 3,5-bis(1,1-dimethylethyl)- (5.950%); 4-O-methylmannose (4.085%) and n-hexadecanoic acid (8.733%).

Previous study found other major compounds on red *C. vespertilionis* leaves extract such as 10-undecenoic acid (5.00%), 6-methylheptyl-2-propenoate (4.96%), 2-(2-benzothiazolylthio)-1-(3,5-dimethylpyrazolyl)-ethanone (2.70%), tetrahydro-2-methyl-thiophene (61.77%) and phytol (8.59%) [11]. Another study also found that *C. vespertilionis* leaves methanolic extract exhibited hexadecanoic acid (4.87 %), 9,12,15-octadecatrienoic acid (7.82%), phytol (6.97%), 2-propenoic acid (8.75%) and cyclododecane (7.04%) [12]. These previous study outcomes also can be found in Table 2 such as 9,12,15-octadecatrienoic acid, phytol and hexadecanoic acid.

Table 1. Conditions of samples

Plant	Technique	Solvent	Sample Code	Temperature (°C)	Time (hours)
Red *C. vespertilionis*	Maceration	MeOH	RMM	40	48
		EtOH	RME		
	Soxhlet extraction	MeOH	RSM	< 64.7	5
		EtOH	RSE	< 78.37	


(a)
Abundance (%)
81.967
4.246
7.778
Retention time (min)
(b)
Abundance (%)
27.414
10.247
Retention time (min)
(c)
Abundance (%)
31.037
11.655
21.491
Retention time (min)

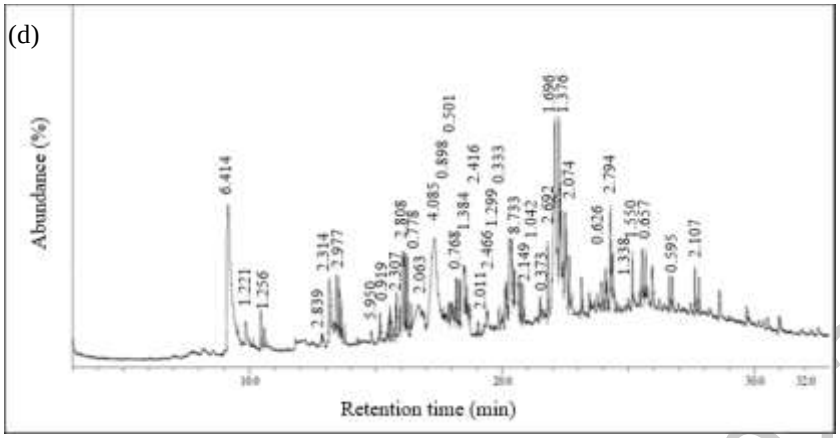


Figure 1. GC-MS chromatogram abundance (%) versus retention time (min) of a) red *C. vespertilionis* methanolic leaves extract in maceration (RMM); b) red *C. vespertilionis* ethanolic leaves extract in maceration (RME); c) red *C. vespertilionis* methanolic leaves extract in Soxhlet extraction (RSM); d) red *C. vespertilionis* ethanolic leaves extract in Soxhlet extraction (RSE)

Table 2. Phytochemicals compounds found in red *C. vespertilionis* leaves based on abundance and retention time

	Bioactive Phytochemicals	**Retention Time (min)**	**Abundance (%)**			
			Maceration		**Soxhlet**	
			MeOH (RMM)	**EtOH (RME)**	**MeOH (RSM)**	**EtOH (RSE)**
1	Acetic acid, butyl ester	3.683	81.967	-	11.655	-
2	1-butanol, 3-methyl-, acetate	9.179	4.246	27.414	31.037	6.414
3	4H-pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	9.815	0.551	2.533	3.694	1.221
4	1-dodecene	10.459	-	-	-	1.256
5	3-tetradecene, (Z)-	10.461	-	1.657	-	-
6	Dodecane	10.590	-	0.975	-	-
7	1,3-diisobutyrin, trimethylsilyl	11.817	1.886	-	1.537	-
8	Heptanoic acid, propyl ester	13.140	0.497	5.700	-	-
9	Hexanoic acid, 3-oxo-, ethyl ester	13.160	-	0.456	5.549	2.839
10	1-tridecene	13.446	0.114	3.924	-	-
11	1-tetradecene	13.449	-	-	0.127	2.314
12	Tetradecane	13.555	-	2.295	0.221	2.977
13	Cyclotetradecane	13.775	-	0.214	-	-
14	Phenol, 3,5-bis(1,1-dimethylethyl)-	15.150	0.779	6.232	0.578	5.950
15	2-hexyl-1-octanol	15.570	-	0.394	-	-
16	1-octanol, 2-butyl-	15.801	-	0.526	-	-
17	1-hexadecene	16.072	0.145	0.328	-	-

Table 2 (cont'd). Phytochemicals compounds found in red *C. vespertilionis* leaves based on abundance and retention time

	Bioactive Phytochemicals	Retention Time (min)	Abundance (%)			
			Maceration		Soxhlet	
			MeOH (RMM)	EtOH (RME)	MeOH (RSM)	EtOH (RSE)
18	1-octadecene	16.077	-	6.079	-	0.919
19	1-heptadecene	16.079	-	-	0.115	2.307
20	Pentadecane	16.162	0.123	1.865	-	-
21	Nonadecane	16.167	-	-	-	2.808
22	Hexadecane	16.170	0.202	-	0.147	-
23	Dichloroacetic acid, 4-hexadecyl ester	16.253	-	-	-	0.778
24	2-undecene, 3-methyl-, (Z)-	16.254	-	1.019	-	2.063
25	1,2-benzenedicarboxylic acid, mono(2-ethylhexyl) ester	16.269	-	-	0.321	-
26	4-O-methylmannose	17.404	7.778	-	21.491	4.085
27	.alpha.-d-mannofuranoside, methyl	17.421	-	10.247	-	-
28	1-hexanol, 5-methyl-2-(1-methylethyl)-	17.958	-	0.231	-	-
29	Cyclohexane, 2-butyl-1,1,3-trimethyl-	18.173	-	-	-	0.898
30	1-decanol, 2-hexyl-	18.176	-	0.510	0.042	0.501
31	1-pentadecene, 2-methyl-	18.214	-	-	-	0.768
32	Cyclopropane, 1-methyl-1-(1-methylethyl)-2-nonyl-	18.400	-	-	-	1.384
33	2-undecene, 9-methyl-, (E)-	18.430	-	5.505	-	-
34	1-heneicosanol	18.431	-	2.156	-	2.416
35	4,5-heptadien-2-ol, 3,3,6-trimethyl-	18.435	-	-	0.028	-
36	n-heneicosane	18.501	-	-	-	2.011
37	Undecane, 3-methylene-	18.594	-	-	-	2.466
38	Tridecane, 3-methylene-	18.595	-	1.300	-	1.299
39	2-pentadecanone, 6,10,14-trimethyl-	19.039	-	-	0.054	0.333
40	1,2-benzenedicarboxylic acid, bis(2-methylpropyl) ester	19.392	-	-	0.330	-
41	Hexadecanoic acid, methyl ester	19.895	-	-	0.105	-
42	Heptacosanoic acid, methyl ester	19.900	-	-	0.205	-
43	1-eicosanol	20.282	-	-	2.888	-
44	Methyl di-tert-butylhydroxyhydrocinnamate	20.284	-	-	2.471	-
45	n-hexadecanoic acid	20.291	0.772	3.090	4.465	8.733
46	Dibutyl phthalate	20.392	-	-	0.974	-
47	Octacosanol	20.558	-	0.804	-	2.149
48	1-octadecanol	20.561	0.048	4.152	-	-
49	3-eicosene, (E)-	20.562	-	-	0.051	-

Table 2 (cont'd). Phytochemicals compounds found in red *C. vespertilionis* leaves based on abundance and retention time

	Bioactive Phytochemicals	Retention Time (min)	Abundance (%)			
			Maceration		Soxhlet	
			MeOH (RMM)	EtOH (RME)	MeOH (RSM)	EtOH (RSE)
50	Heneicosane, 11-(1-ethylpropyl)-	20.607	-	-	-	1.042
51	1-docosene	20.816	-	0.345	-	-
52	1-tetradecanol	21.502	-	-	-	0.373
53	Phytol	21.798	0.480	1.775	0.488	
54	1,1,1,3,5,7,7,7-octamethyl-3,5-bis-(trimethylsiloxy)-tetrasiloxane	21.808	-	-	-	2.692
55	Methyl 9-methyltetradecanoate	21.901	-	-	0.131	-
56	9,12,15-octadecatrienoic acid, methyl ester, (Z,Z,Z)-	22.090	-	-	1.379	-
57	9,12,15-octadecatrienoic acid, (Z,Z,Z)-	22.092	0.267	1.343	2.365	1.696
58	Tetradecyl trifluoroacetate	22.253	-	-	1.035	-
59	Octadecanoic acid	22.255	0.146	0.500	1.608	1.376
60	1-tetracosanol	22.498	-	-	-	2.074
61	2,4-pentadien-1-ol, 3-pentyl-, (2Z)-	22.659	-	-	0.206	-
62	9-(3,3-dimethyloxiran-2-yl)-2,7-dimethylnona-2,6-dien-1-ol	22.660	-	0.753	-	-
63	1-dodecanol, 2-octyl-	24.091	-	-	-	0.626
64	Heptasiloxane, hexadecamethyl-	24.377	-	-	-	2.794
65	Hexacosane	25.151	-	-	-	1.338
66	Diisooctyl phthalate	25.679	-	-	0.472	1.550
67	Docosane	25.954	-	-	-	0.657
68	Eicosane	26.730	-	-	-	0.595
69	Squalene	27.788	-	-	-	2.107
70	Cholesta-4,6-dien-3-ol, (3.beta.)-	30.174	-	-	0.068	-
71	.alpha.-tocopheryl acetate	30.522	-	-	0.034	-
	Quantity of identified compound		16	30	33	37

Optimisation of extraction

Correlation was used between abundance and retention time of the sample in GC-MS for all compounds found. Maceration using MeOH (RMM) was significantly high at $P < 0.011$ (Figure 2). When using EtOH (RME), the significant difference was at $P < 0.049$. In Soxhlet, the significant difference was high in MeOH (RSM) with $P < 0.009$ and there was no significant difference in (RSE) which was at $P < 0.104$. The smaller the P value, the more significant it is. A total of seventy-one (71) abundance were analysed for each sample. The results indicated that significant difference between abundance and retention time increased when using Soxhlet extraction particularly in MeOH.

Extraction methods using different solvents play an important role to exhibit bioactive phytochemicals. Based on Figure 2, abundances of compounds are exhibited more when using Soxhlet extraction and MeOH. A previous study stated that the polarity of MeOH could be the reason for the high exhibition of

compounds [13]. Other study stated that using MeOH produces high bioactive phytochemicals particularly compounds that cure inflammation which is extracted from the branches of *S. buxifolia* [14]. It was also revealed that MeOH is a solvent that is more effective in extracting secondary metabolite compounds from *M. viridis* than any other plants in the Lamiaceae family [15].

The technique of extraction is also one of the factors that contribute to the effectiveness of bioactive phytochemicals attribution. Based on Figure 2, more compounds were exhibited using Soxhlet extraction as compared to maceration. Soxhlet extraction offered more efficiency and was less time-consuming as compared to maceration. Other researches obtained similar results in *Nepeta leucophylla* and *Potentilla atrosanguinea* aerial parts when using Soxhlet extraction where it exhibited significantly higher, and attained maximum percentage yields than maceration [4, 16]. Therefore, for red *C. vespertilionis* leaves, Soxhlet extraction obtained highest compounds attribution as compared to the maceration technique. Overall, red *C. vespertilionis* leaves work efficiently using Soxhlet extraction and MeOH as a solvent.

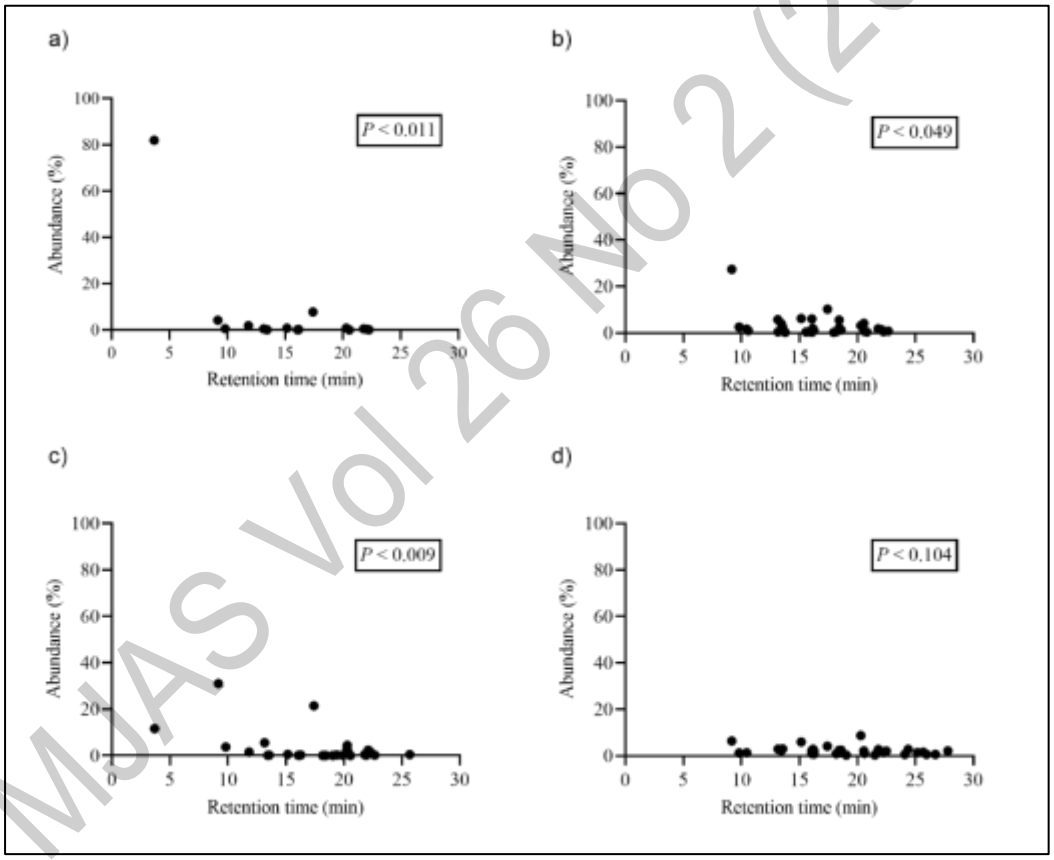


Figure 2. Correlation between abundance (%) versus retention time (min) of a) RMM b) RME c) RSM and d) RSE

Major bioactive phytochemicals

Four (4) samples (RMM, RME, RSM and RSE) of *C. vespertilionis* leaves extract, detected seventy-one (71) bioactive phytochemicals, which consisted of major and minor compounds using GC-MS, as presented in Table 2. The abundance presented for each sample is shown in Figure 1. Through this analysis, it was found that eleven (11) major compounds achieved higher than

4 % of the abundance from all of samples as presented in Table 3.

The eleven (11) major bioactive phytochemicals were acetic acid, butyl ester (81.967%), 1-butanol, 3-methyl-, acetate (31.037%), heptanoic acid, propyl ester (5.700%), hexanoic acid, 3-oxo-, ethyl ester (5.549%), phenol, 3,5-bis(1,1-dimethylethyl)- (6.232%), 1-octadecene (6.079%), 4-O-methylmannose (21.491%), .alpha.-d-mannofuranoside, methyl (10.247%), 2-undecene, 9-methyl-, (E)- (5.505 %), n-hexadecanoic acid (8.733%), and 1-octadecanol (4.152%).

The n-hexadecanoic acid and 1-octadecanol possessed anti-inflammatory activities [17,18]. The antimicrobial agent was revealed by 1-butanol, 3-methyl-, acetate, [19]. Ethyl acetate root extract of *C. vespertilionis* showed the highest total phenolic content that is responsible for good anti-inflammatory and anti-microbial properties [20,21]. Meanwhile, 1-octadecene possessed anti-cancer property [22, 23]. Based on the previous study, *C. vespertilionis* extract has viability against various cell lines such as WRL68 (normal liver), CRL 2522 (fibroblast), HepG2 (liver carcinoma), MCF-7 (breast cancer) and HaCaT (keratinocyte) with the IC50 values of 1.93, 1.51, 1.63, 1.74 and 1.22 mg/ml, respectively [24]. Four (4) compounds had antibacterial activities that included n-hexadecanoic acid, 4-O-methylmannose, 1-octadecene and phenol, 3,5-bis(1,1-dimethylethyl)- [23, 24, 26-30].

Most of the compounds possessed anti-oxidants, including 1-octadecanol, n-hexadecanoic acid and 1-octadecene [18, 23, 26, 31]. *C. vespertilionis* ethyl acetate: methanol extract has significantly exhibited the highest DPPH scavenging activity with the IC50 value of 0.549 ± 0.02 mg/mL [32]. Acetic acid, butyl ester was the most abundant compounds that is beneficial as an antifungal and antitumour [33]. *C. vespertilionis* inhibited tumour growth and increase survival time of mice induced with S180 AND H22 tumour cells [34]. Another known compound was phenol, 3,5-bis(1,1-dimethylethyl)- which acts as an antiseptic, disinfectant and flavourant [24, 29]. No biological activity was reported for heptanoic acid, propyl ester; hexanoic acid, 3-oxo-, ethyl ester; .alpha.-d-mannofuranoside, methyl and 2-undecene, 9-methyl-, (E)-. Therefore, only seven (7) out of eleven (11) major compounds were reported their biological activities.

Table 3. Major bioactive phytochemicals of red *C. vespertilionis* leaves

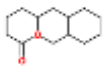
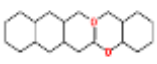
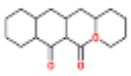
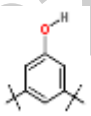
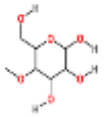
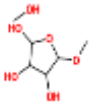
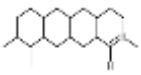
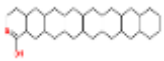
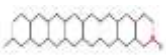
	Name of Compound/ Chemical Classes	Rt (Min)	Abundance (%) Maceration		Soxhlet		Formula	MW	Structure	Biological Properties
			MeOH (RMM)	EtOH (RME)	MeOH (RSM)	EtOH (RSE)				
1	Acetic acid, butyl ester (Carboxylic acid ester)	3.683	+ 81.967	-	+ 11.655	-	$C_{16}H_{12}O_2$	116		Antifungal, Antitumor [33]
2	1-butanol, 3-methyl-, acetate (Carboxylic acid ester)	9.179	+ 4.246	+ 27.414	+ 31.037	+ 6.414	$C_7H_{14}O_2$	130		Antimicrobia [19]
3	Heptanoic acid, propyl ester (fatty acid ester)	13.140	-	+ 5.700	-	-	$C_{10}H_{20}O_2$	172		No activity reported

Table 3 (cont'd). Major bioactive phytochemicals of red *C. vespertilionis* leaves

	Name of Compound/ Chemical Classes	Rt (Min)	Abundance (%) Maceration		Soxhlet		Formula	MW	Structure	Biological Properties
			MeOH (RMM)	EtOH (RME)	MeOH (RSM)	EtOH (RSE)				
4	Hexanoic acid, 3-oxo-, ethyl ester (fatty acid ester)	13.160	-	-	+ 5.549	-	$C_8H_{14}O_3$	158		No activity reported
5	Phenol, 3,5-bis(1,1-dimethylethyl)- (phenolic ester)	15.150	-	+ 6.232	-	+ 5.950	$C_{14}H_{22}O$	206		Antiseptic, disinfectant, flavouring, antibacterial [24,29]
6	1-octadecene (Alkene hydrocarbon)	16.077	-	+ 6.079	-	-	$C_{18}H_{36}$	252		Antibacterialal, antioxidant, anticancer [22, 23, 26]
7	4-O-methylmannose (aliphatic ether alcohol)	17.404	+ 7.778	-	+ 21.491	+ 4.085	$C_7H_{14}O_6$	194		Antibacterial [27, 28]
8	alpha.-d-mannofuranoside, methyl (methyl mannoside)	17.421	-	+ 10.247	-	-	$C_7H_{14}O_6$	194		No activity reported
9	2-undecene, 9-methyl-, (E)- (hydrocarbon)	18.430	-	+ 5.505	-	-	$C_{12}H_{24}$	168		No activity reported
10	n-hexadecanoic acid (fatty acid)	20.291	-	-	+ 4.465	+ 8.733	$C_{16}H_{32}O_2$	256		Anti-inflammatory, antibacterial, antioxidant [17, 30, 31].
11	1-octadecanol (fatty alcohol)	20.561	-	+ 4.152	-	-	$C_{18}H_{38}O$	270.5		Anti-inflammatory, antioxidant [18].
	Total of major compounds		3	7	5	4				

Conclusion

This study revealed that red *C. vespertilionis* leaves have various bioactive compounds that has high potential to treat diseases. Red *C. vespertilionis* leaves possessed biological activities of anti-inflammatory, antitumour, anticancer, antibacterial, antimicrobial, antioxidant and antifungal properties. The compounds were greatly exhibited from Soxhlet extraction and MeOH as a solvent. Conclusively, this study achieved success in identifying bioactive phytochemicals of red *C. vespertilionis* leaves in different techniques and solvents. Further study needs to be done to isolate bioactive phytochemicals and investigate its biological activities. A comparison to green *C. vespertilionis* needs to be analysed to observe the differences in terms of bioactive phytochemicals and biological activities.

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