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Chemical Profiling and Antioxidant Properties of Leaf and Stem Extracts of *Christia vespertilionis*

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Christia vespertilionis (CV) (L. f.) Bakh. f. (Fabaceae), or known as 'Green Butterfly Wing,' is gaining popularity as a valuable, underutilized medicinal plant having antioxidant properties as well as being a new potential source of natural products. The main objectives of this study are to identify the chemical profile of leaf and stem extracts of *Christia vespertilionis* (CV) using Fourier transform infrared (FTIR) spectroscopy and to evaluate its antioxidant properties (i.e total phenolic content (TPC), total flavonoid content (TFC), and 2,2-diphenyl-1-pierylhydrazyl (DPPH) free radical scavenging ability). The extraction of the plant was conducted using polar to non-polar solvents which are water, methanol, ethyl acetate, chloroform and hexane. FTIR spectroscopy analysis of CV revealed the presence of functional group of -OH, C-H, C=O, C=C, C-O, and N-H that are mainly attributed to phenolics and flavonoids. The bioassays result of the leaf and stem extracts showed a significant quantity of phenolic compounds, ranging from 1.52 to 5.82 mg of GAE/g extract. Moreover, the leaves and stems also showed significant number of flavonoid compounds, ranging from 3.45 to 27.57 mg of QE/g extract. The DPPH radical scavenging activity for leaf and stem showed higher in water extract (72%) followed by chloroform (37%), methanol (36%), ethyl acetate (24%) and hexane (17%) extracts. The outcome of the study will provide the information on the chemical profile and antioxidant properties CV that can be used for potential applications on diverse medicinal and bioactive components attributes.

Key words: *Christia vespertilionis*; Chemical profiling; FTIR; Antioxidant

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Herbalism, or the use of medicinal plants, is not a new practice. Potential therapeutic plants are thought to have high antioxidant levels, which contribute to their anti-cancer and anti-inflammatory properties, particularly in the case of breast cancer [1]. Flavonoids, tannins, curcumin, and stilbene resveratrol are just a few examples of the polyphenolic compounds found in plants that have attracted the interest of scientists for their medicinal effects and the methods by which they work. They are known to be naturally present anti-oxidants and have been linked to cytotoxic activity [2].

Butterfly pea is indeed a decoction of Mariposa *Christia vespertilionis* leaves that is broadly distributed by cancer sufferers across the whole of Malaysia. These have gained immense popularity among all Malaysians, not just cancerous, but also investigators who are trying to figure out what is this plant's true potential [3].

Considerable evidence has emerged indicating that reactive oxygen species (ROS) and other oxidants play a significant part in the development of a variety of disorders and illnesses [4]. Flavonoid components are usually located in plants' flowers, leaves, and seeds. Plant flavonoids, as secondary metabolites, play vital roles in facilitating plant responses to bio-logical and non-biological environmental exposures [5].

Metabolite analysis, especially metabolite profiling throughout biological systems matrices, is critical throughout many fields of life science [6]. It necessitates a streamlined extraction, isolation, and analysis pipeline such that a significant number of metabolites can be measured in a single experiment, a powerful and quantifiable method in the existence of a phenomenally chemically complex combination (the matrix) discovered in cellular crude extract [7]. To accomplish, or even approach, this goal, a variety of

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of analytical techniques must be used like Fourier transform infrared (FTIR).

EXPERIMENTAL

Chemicals and Materials

Chemical Reagents

Sodium nitrate, potassium chloride, 2, 2-diphenyl-1-picrylhydrazyl (DPPH), Folin–Ciocalteu reagent, standards of phenolic and flavonoid compounds (gallic acid and quercetin), sodium carbonate, sodium hydroxide, methanol, ethyl acetate, chloroform, and aluminium chloride were obtained from E-merck.

Sample Collection and Preparation

About 2 kg of dried plant material (leaves and stems) was obtained from local farmer in Perak, Malaysia. About 2 g of grounded plant material was added with 40 mL of solvent of different polarities (hexane, chloroform, ethyl acetate, methanol, water). Then, the mixture was sonicated at room temperature for 45 min. The mixture was filtered and concentrated using rotary vacuum evaporator. Following this, all crude extracts were lyophilised to remove excess water. All extracts were kept in refrigerator (4 °C) until further analyses. Six biological replicates were performed for different parts (leaf and stem) and different solvent polarities of extraction.

Characterization Methods

2, 2- Diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging Assay

The free radical scavenging activity of *Christia vespertilionis* leaves and stems extracts was tested using 2,2-diphenyl-1-picrylhydrazyl (DPPH) according to [8]. 0.5 mg of each solvent extract was weighed and dissolved in methanol. Sonication and vortexing homogenized the samples. Accurately, 100 µL of methanol was pipetted into every 96 wells plate and treated with 100 µL of sample for the first row and produce dilution, then discard the last 100 µL. 100 µL of DPPH was added in all wells (2.4 mg in 50 mL of methanol). The test plate was darkly incubated for 30 min. A microplate reader measured the absorbance at 517 nm of quercetin, a positive control.

$$\% \text{ inhibition} = [(AB-AS)/AB] \times 100$$

where,
AB and AS are the absorbance of reagent blank and tested samples, respectively.

The results are reported as mean ± standard deviation of the percentage inhibition against DPPH at the concentration of 500 µg/mL. Gallic acid was used as a positive control [9]

Total Phenolic Content (TPC)

The total phenolic content of the extracts was quantified using Folin-Ciocalteu reagent method. Extracts were dissolved in methanol (1 mg/mL). 100 µL of Folin-Ciocalteu reagent (5 min incubation) and 80 µL of 7.5% (w/v) sodium bicarbonate (Na_2CO_3) were added to 20 µL of extract. The mixture was incubated for at least 30 minutes to 2 hr at room temperature and the absorbance at 760 nm was measured using a spectrophotometer. Eight points calibration curves were plotted with 8 concentrations of gallic acid [10].

Total Flavonoid Content (TFC)

The procedure described was used with slight modification [10]. 20 µL of extract stock (1 mg/mL in methanol), 120 µL distilled water, 10 µL sodium nitrate (5%) then 5 minutes incubation, 10 µL 10% aluminum chloride then 6 minutes incubation, 40 µL sodium hydroxide (1 mol/L) were added in well. Then, 15 minutes of incubation at room temperature before reading at 510 nm.

UV-Vis Spectrophotometry

By determining the type of electronic transition of compounds in the wavelength range of 300-800 nm, the UV-Vis spectrophotometer UV-1900i (Shimadzu cooperation 80636) was used to screen the phyto-chemicals from *Christia vespertillionis* extracts.

Metabolite Profile

ATR-FTIR Analysis

20 mg of crude extracts re-dissolve with 1 mL of analytical acetone were analyzed using Shimadzu Corp IR Tracer-100 FTIR spectrometer equipped with 40 interferograms and a scan range of 600 to 4000 cm^{-1} at a resolution of 4 cm^{-1}. ATR crystal center was loaded with about 0.1 mL of the sample (1 mg/mL). At room temperature, samples were dried for approximately 40 sec. A spectrum of the ATR crystal was recorded before each sample scan using the same instrumental conditions as the background. After each sample scan, the ATR plate was cleaned with acetone and dust-free tissue. Six biological replicates and three technical replicates were used for the FTIR analysis of the samples. All FTIR values were transformed to ASCII files and multivariate data analysis was performed.

Multivariate Data Analysis (MVDA)

The SIMCA-P software (v. 14.1, Umetrics, Ume, Sweden) was used for multivariate data analysis (MVDA), including component analysis (PCA). The PCA is a technique for reducing the dimensionality of such datasets in order to improve interpretability while

minimizing information loss. It is typically used for metabolite fingerprinting and classification [11].

RESULTS AND DISCUSSION

DPPH Activity of *Christia vespertilionis*

The DPPH free radical scavenging technique involves the discoloration of a molecule as a consequence of its reaction with the stable radical DPPH (max=517 nm), which is produced when a molecule or antioxidant with weak A-H bonding interacts with DPPH. Colour shifts from purple to yellow signal a reduction in DPPH radical absorbance. To produce a stable diamagnetic molecule, DPPH may receive either an electron or a hydrogen radical, resulting in greater water content, ~~in~~ Table 1, for both leaf and stem extracts containing a hydroxyl group.

Total Phenolic Content (TPC)

The solubility of phenolic compounds in the extraction solvent affects the recovery of polyphenols from plant sources. [12]. The solvents utilized in this work can be categorized by the degree of polarity based on their dielectric constant. The maximum percentage and total phenolic content extractable were determined in ethyl acetate for the leaf part and methanol for the stem as shown in Table 1. Polyphenols are frequently more soluble in organic solvents that are less polar than water [12]. The total phenolic content of the hexane extracts, on the other hand, was found to be the lowest. As a result, the total phenolic content was higher in polar solvent extracts and lower in non-polar solvent extracts.

Total Flavonoid Content (TFC)

The total flavonoid content (TFC) of *Christia vespertillionis* extracts as shown in Table 1 was determined using the aluminium chloride ($AlCl_3$) assay. Due to the increased number of chlorophyll, which adds to the overall quantity of flavonoids as revealed in UV-vis has a higher wavelength range of 668-695 nm, leaf hexane and stem chloroform exhibit higher flavonoid content. The structure of the flavonoids determines whether or not they are polar or non-polar [13]. Tocopherols are an example of a molecule that is soluble because of its long aliphatic chain. Because of this, an extract made using hexane and chloroform could include flavonoids in it.

Table 1. TFC, TPC and DPPH activity of *Christia vespertilionis*

Part	Solvents	TFC (mg of QE/g sample)	TPC (GAE/g sample)	DPPH Activity (at 500µg /mL)
Leaf	Hexane	27.57± 0.070	1.86±0.32	17.24±0.01
	Chloroform	19.45± 0.034	1.52±0.14	37.97±0.02
	Ethyl acetate	14.15± 0.041	2.69±0.39	24.94±0.01
	Methanol	8.02± 0.002	2.45±0.24	36.70±0.02
	Water	3.45± 0.006	1.65±0.18	72.97±0.02
Stem	Hexane	9.74± 0.015	0.86±0.20	7.65±0.013
	Chloroform	12.27± 0.017	1.03±0.06	43.84±0.004
	Ethyl acetate	11.25± 0.015	1.14±0.10	47.69±0.005
	Methanol	7.40± 0.033	4.22±0.22	69.88±0.003
	Water	6.10± 0.011	1.49±0.15	76.54±0.002

a) TFC Leaf b) TPC Leaf c) DPPH Leaf

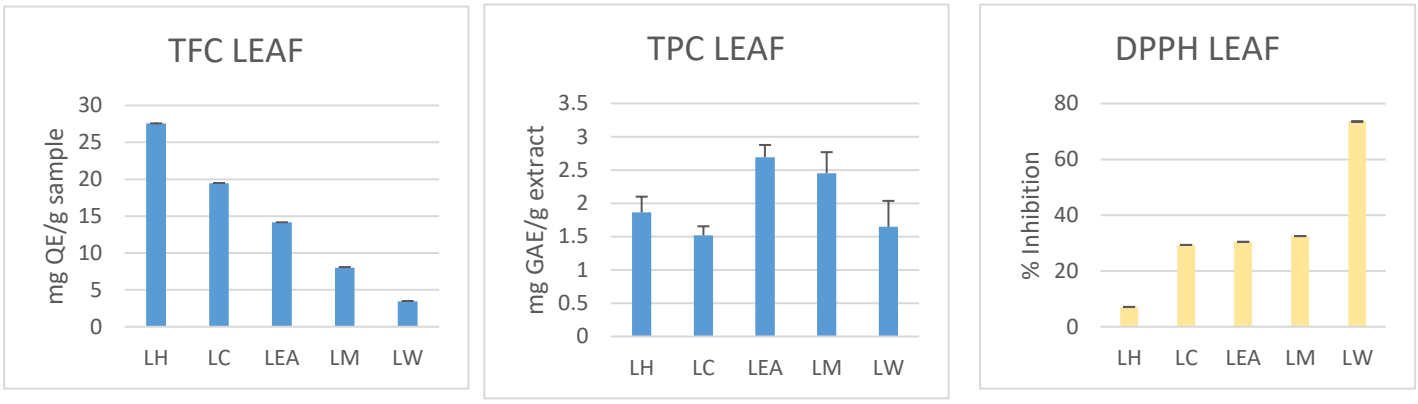

Figure 1. Bioassay (a)TFC (b) TPC and (c) DPPH of leaf extracts

a) TFC Stem b) TPC Stem c) DPPH Stem

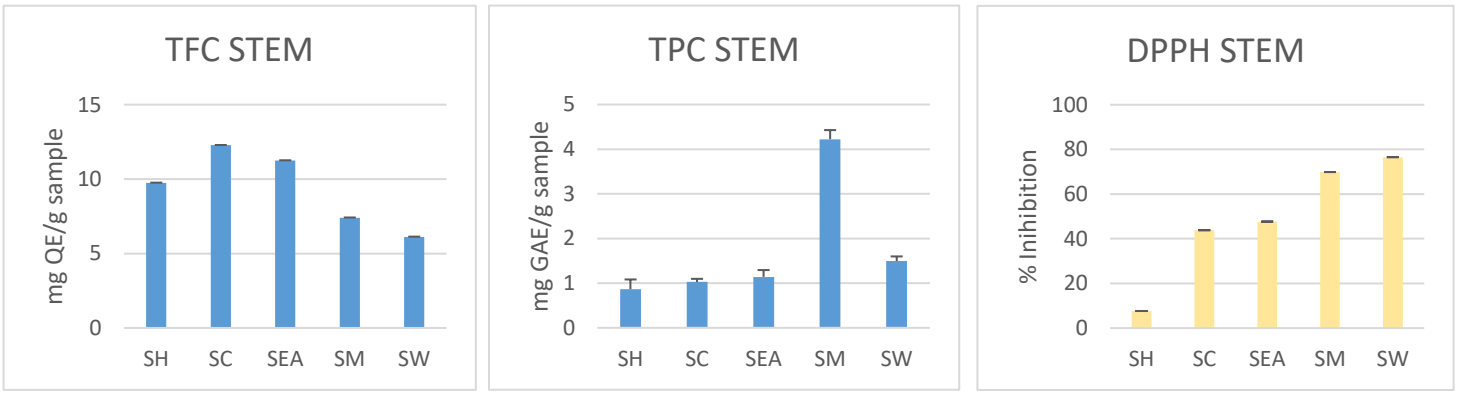

Figure 2. Bioassay (a)TFC (b) TPC and (c) DPPH of stem extracts

Screening of Phytochemicals from *Christia vespertillionis* extracts using UV-Vis Analysis

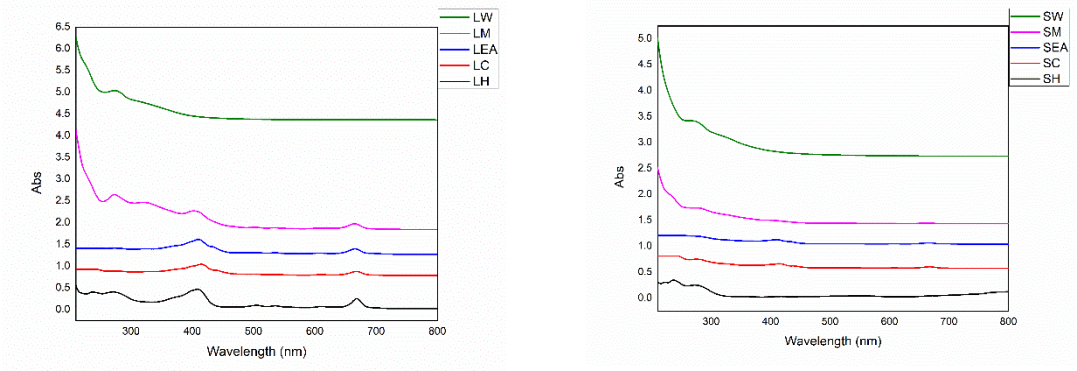

Figure 3. UV-Vis spectra of (a) left extract and (b) stem extract of *Christia vespertillionis*

The UV-Vis spectrophotometry, Figure 3, was performed for the identification of chromophore groups and aromatic rings present in the extract of *Christia vespertillionis* containing electronic transition of π-bonds, σ- bonds, and lone pair of electrons as well [14]. Due to the strong peaks and suitable baseline, the

qualitative UV-Vis profile of *Christia vespertillionis* solvent extracts was collected at 300–800 nm. Tables 2 and 3 indicate leaf and stem absorption peaks. *Christia vespertillionis* extract absorption spectra are practically clear in the wavelength range of 300-800 nm as shown in Figure 3. The absorption bands observed are displayed in Table 2. One or more peaks in the UV-Vis spectra from 200-400 nm indicate unsaturated groups and heteroatoms like S, N and O. [15]. Some flavonoids have an additional absorbance maximum between 300 and 550 nm. The spectrum shows a peak at position 400-500 nm which confirms the presence of organic chromophores within *Christia vespertillionis* extract. According to [16] peaks region around 663 nm showed chlorophylls. The absorption spectra of *Christia vespertillionis* extracts are practically clear in the wavelength range of 668-695 nm.

Table 2. Absorption profile for leaf extracts

Leave hexane		Leave chloroform		Leave ethyl acetate		Leave methanol		Leave water	
Wavelength (nm)	Abs.	Wavelength (nm)	Abs.	Wavelength (nm)	Abs.	Wavelength (nm)	Abs.	Wavelength (nm)	Abs.
695	0.041	668	0.095	666	0.127	665	0.133	791	0.003
669	0.201	610	0.02	607	0.028	608	0.032	273	0.678
610	0.037	538	0.026	534	0.037	534	0.039		
559	0.025	500	0.032	505	0.041	503	0.047		
533	0.048	415	0.261	473	0.038	402	0.426		
504	0.061	329	0.094	410	0.344	319	0.624		
470	0.059	274	0.105	324	0.128	273	0.803		
409	0.45			273	0.143				
319	0.141								
269	0.209								

Table 3. Absorption profile for stem extracts

Stem hexane		Stem chloroform		Stem ethyl acetate		Stem methanol		Stem water	
Wavelength (nm)	Abs.	Wavelength (nm)	Abs.	Wavelength (nm)	Abs.	Wavelength (nm)	Abs.	Wavelength (nm)	Abs.
669	0.021	668	0.034	666	0.127	664	0.021	265	0.68
534	0.011	609	0.011	607	0.028	604	0.013		
504	0.012	539	0.014	534	0.037	498	0.02		
409	0.042	506	0.015	505	0.041	273	0.312		
273	0.058	415	0.093	473	0.038				
220	0.155	275	0.18	410	0.344				
				324	0.128				

Characterization

Fourier transforms infrared (FTIR) of *Christia vespertilionis*

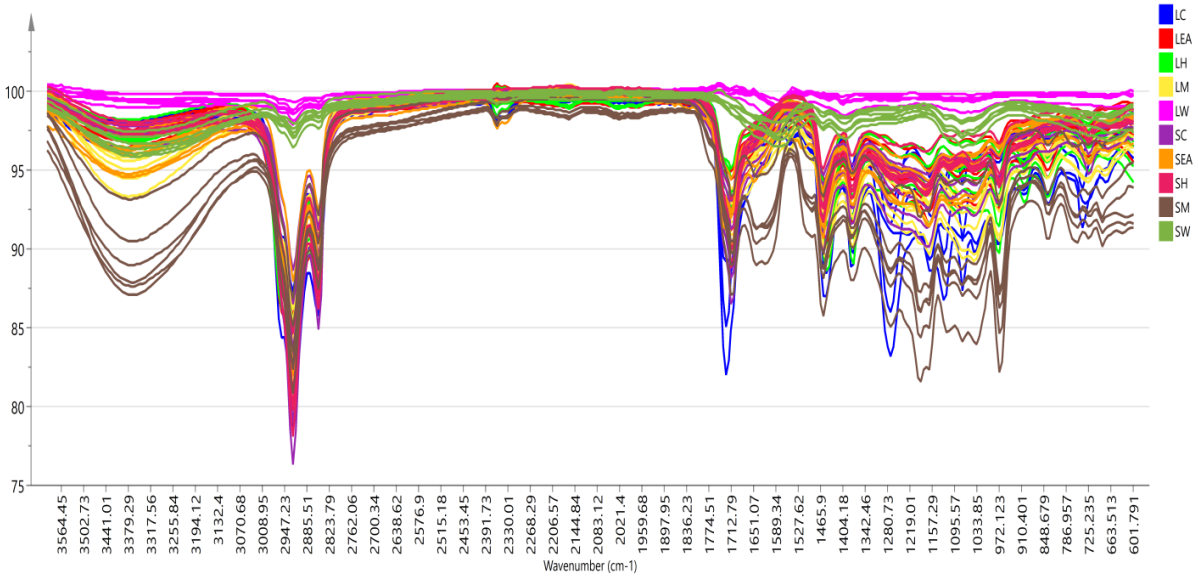

Figure 4. ATR-FTIR spectra overlay of *Christia vespertilionis*

This research examined C. *vespertilionis* (CV) extract functional groups using FTIR. Figure 4 displays the CV extract FTIR graph. Table 4 lists the wavenumber (cm^{-1}), assignment, type of vibration, and main attribution of FTIR spectra. The O-H bonded alcohol, phenol, and carboxylic acid peak was 3379 cm^{-1}. Saturated (sp^3) carbon from 2885 to 2947 cm^{-1} exhibited the C-H stretch of alkanes, alkenes, aromatics, and aldehydes. Aldehydes and esters have C=O at 1712 cm^{-1}. From 1527 to 1651 cm^{-1} showed alkene, primary and secondary amine, and amide of C=C and N-H bending. Alkane C-H occupied at range from 1342 to 1465 cm^{-1} [15]. Alcohols, ethers, esters, carboxylic acid, and anhydrides with C-O wavenumbers from 1033 to 1280 cm^{-1}. C-H out of plane bend at 601-848 cm^{-1}.

Table 4. Tentative identification of compounds in *Christia vespertilionis* based on FTIR data

Wavenumber (cm^{-1})	Assignment	Type of Vibration	Tentative Assignment	Reference(s)
3379	O-H	Stretch	Alcohol, phenols, carboxylic acid	Murugesu *et al.*, 2020
2885-2947	C-H	Stretch	Alkanes, alkenes, aromatic, aldehydes	Jain *et al.*, 2016
1728	C=O		Aldehydes, ester	
1651	C=C. N-H	Bend	Alkene, primary and secondary amines and amide	Murugesu *et al.*, 2020
1342-1465	C-H		Alkanes	Murugesu *et al.*, 2020
1033-1280	C-O		Alcohols, ethers, esters, carboxylic acid, anhydrides	Murugesu *et al.*, 2020
601-848	C-H	Out of plane bend	Aromatic	Jain *et al.*, 2016

Multivariate Data Analysis (MVDA)

Principal Component Analysis (PCA)

a) Leaf extracts b) Stem extracts

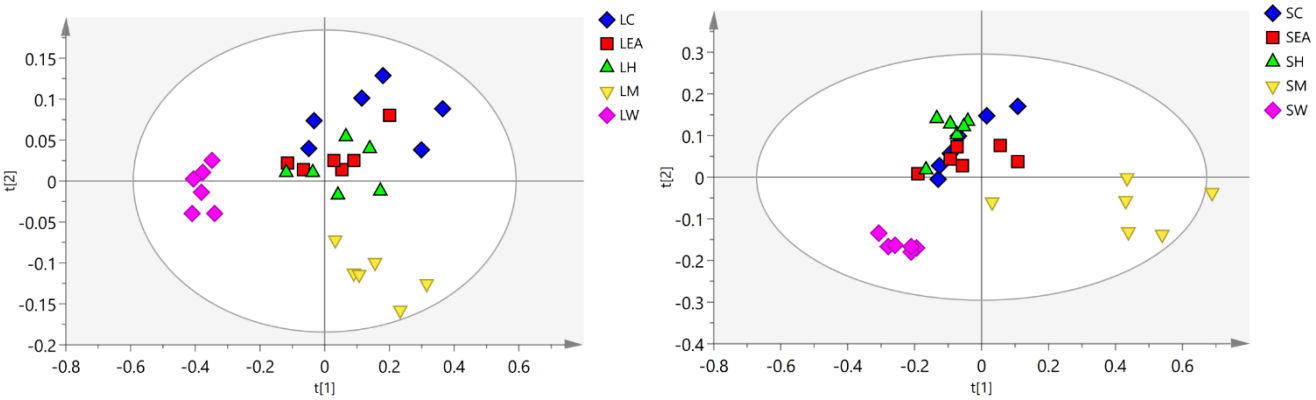

Figure 5. Principal component analysis (PCA) scores plot of (a) leaf extracts and (b) stem extracts

Determination of FTIR fingerprints of different extracts is challenging, therefore the MVDA via the principal component analysis (PCA) was used to further identify the chemical profile that related to different extraction solvents. Figure 5 shows the PCA scores plot of leaf and stem extracts. The PCA of leaf extracts (Figure 5a) showed top two PCs explained by 84.1% of the variance. The R2X (cum) and Q2 (cum) values are 0.998 and 0.991 make the PCA an excellent model. The PCA of stem extracts showed top two PCs of 88.8% variance. The later showed R2X (cum) and Q2 (cum) values of 0.998 and 0.992 giving the PCA an excellent model. The PCA scores plot showed three distinctive clusters of leaf and stem extracts of green *Christia vespertilionis*. At the upper right quadrant showed the clusters of samples extracted by hexane, chloroform, and ethyl acetate, whereas the methanol extracts were situated at the lower right quadrant of PCA scores plot. According to this, the water extracts showed very distinct chemical profile from others that situated at the left quadrant of PCA scores plot. Based on the observation of PCA scores plot, both of different parts were successfully discriminated according to their different solvent polarities of extraction (least to most polar solvents).

Further investigation using their loading scores plot (Figure 6) showed that methanol extracted metabolites having more OH functional group (3394 cm^{-1}) as compare to hexane, chloroform and ethyl acetate. Fingerprint regions (633-995 cm^{-1}) were also the characteristics of methanol extracts. The medium polar extracts (chloroform and ethylacetate) showed the prominent peaks of aldehyde at 1728 cm^{-1} as well as C-H sp^3 at 2924 and 2854 cm^{-1}. The FTIR signals presented in the extracts at 3394, 2854, 2924, and 1728 cm^{-1} were attributed to phenolics and flavonoids. However, since the OH signal is more prominent in methanol extracts it is possible that the solvent extracted more phenolic and flavonoids glycosides as compared to medium polar solvents (chloroform and ethyl acetate). The medium polar solvents most likely extracted the aglycone form [10].

a) Leaf extracts b) Stem extracts

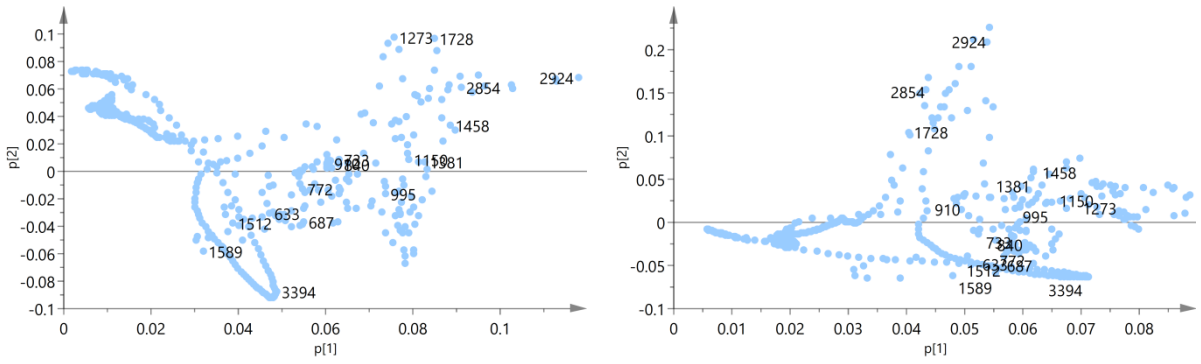

Figure 6. Principal component analysis (PCA) loading scores plot of (a) leaf extracts and (b) stem extracts

CONCLUSION

It is possible that the phenolic and flavonoid compounds in *Christia vespertilionis* contribute to radical scavenging activity and can be regarded as promising plant species for natural sources of antioxidants with potential value for the treatment of many life-threatening diseases. Plant extracts with polyphenol constituents have well-known antioxidant properties due to their ability to contribute hydrogen atoms or electrons and to capture radicals. By inhibiting the branching chain reaction of free radicals, water extracts of the leaf and stem had shown potent antioxidant properties. In this report, we conclude that UV–Vis spectrophotometry and ATR-FTIR helped to capture and screen phytochemical compounds and functional groups that inhibited antioxidants. FTIR analysis in conjunction with MVDA methods could be used to analyze and achieve optimal metabolites data with much less time. As a result, phytoconstituents with a higher concentration of phenolic compounds and flavonoids and good antioxidant activity contribute to the best development of a cost-effective anti-inflammatory medicine with fewer side effects.

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