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Antioxidant Activity and ATR-FTIR-based Chemometric of Wild Erechtites Species Leaves Extracts

Natasha Aziz[1], Basyirah Md Zin[1], and Zalilawati Mat Rashid[1*]

[1]Faculty of Bioresources and Food Industry, Universiti Sultan Zainal Abidin, Besut Campus, 22200 Besut, Terengganu, **Malaysia**

***Correspondence:** zalilawati@unisza.edu.my **Received 25-07-2022, Revised: 31-08-2022, Accepted: 01-09-2022 e-Published: 20-10-2022**

Erechtites sp. belongs to Asteraceae family. *E. valerianifolia* (EV) and *E. hieraciifolia* (EH) are rarely been studied despite the consumption of the leaves as an edible vegetable by local people. Besides, these wild vegetables are being removed and wasted when the habitat was cleaned. In this study, the antioxidant capacity, the phenolics and attenuated total reflection-Fourier transform infrared (ATR-FTIR) spectroscopy analysis of ethanol/water (EW) extracts of both Erechtites were investigated. The leaves were lyophilized, ground and then macerated in EW (4:1) at 50°C (15 minutes), sonicated (15 minutes) and centrifugated. The extract was collected, concentrated using a rotary evaporator, and dried. Four antioxidant assays including 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging, $ABTS^{\bullet+}$ (2,2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical cation, ferric reducing antioxidant power (FRAP), and nitric oxide (NO) scavenging were carried out. The results revealed that EVEW exhibited stronger antioxidant activity as well as more total phenolics (TPC) and flavonoids (TFC), compared to EHEW. The EVEW recorded lower IC_{50} values against DPPH (IC_{50}: 26.32 µg/mL), ABTS (IC_{50}: 20.42 µg/mL) and NO (IC_{50}: 712.24 µg/mL) radicals, whilst higher FRAP level (18.56 mmol Fe^{2+}/g), compared to EHEW. Besides, higher TPC and TFC were determined in EVEW with respective values of 60.00 GAE mg/g gallic acid equivalent and 37.78 mg/g quercetin equivalent. Fourier transform infrared (FTIR) coupled with chemometrics reveals PCA clusters indicating strong chemicals variations infrared signals in area 1 and area 6. Meanwhile, HCA dendrogram classification. Thus, EVEW have potential to be promoted for wider usage besides edible vegetables.

Keywords: *Erechtites valerianifolia*, *Erechtites hieracifolia*, antioxidant activities, ATR-FTIR, chemometrics

INTRODUCTION

Antioxidants are substances that inhibit oxidation of oxidizable chemicals, thus may prevent or delaying some types of cell damage (Brewer, 2011). The natural antioxidants derived from plants including ascorbates, carotenoids, and phenolics have been well reported and recommended in the diet (Khan et al. 2021) due to their protective roles against the oxidative damages (Godic et al. 2014). The scientific interest towards these bioactives, especially the phenolics correspond to their effects in the prevention of oxidative stress associated ailments (Sorice et al. 2016; Mustafa et al. 2021). The phenolics are a large group of secondary metabolites owning aromatic ring(s) with hydroxyl group(s) which are commonly found in fruits, vegetables, cereals, legumes, tea, and coffee (Shahidi and

Ambigaipalan 2015).

Erechtites valerianifolia and *Erechtites hieracifolia* are wild edible vegetables belonging to the Asteraceae family. They are commonly known as 'gedebe lembu' or specifically as 'asang-asang' to the Bidayuh tribe in Sarawak or 'jonggolan' in Indonesia (Batoro et al. 2018). Locally, the young leaves are freshly consumed as 'ulam'. The plants are widely distributed on the lowland, forest, abandoned paddies and crop replantation areas. *E. valerianifolia* has oblong or elliptical blade leaves with many pinnate lobes, while *E. hieraciifolia* has serrated leaves from unlobed to deeply lobes. These plants are considered as weeds and are rarely studied. Despite that, the phytochemicals study was reported decades ago which discussed the hieracifoline and jacobine alkaloids in *E. hieracifolia* (Adams and Gianturco, 1956). Meanwhile, the ethyl acetate extract of *E. valerianifolia* showed the strongest antioxidant activity compared to methanol, hexane, dichloromethane extracts as well as chloroquine (positive control) while also exhibited antifungal and antiplasmodium activities (Puspaningtyas et al. 2018). Few studies found that *E. hieracifolia* extract exhibited antioxidant, antibacterial (Hernández et al. 2013) and antimalarial against various types of Plasmodia (Puspaningtyas et al. 2018).

Thus, this study aims to provide more scientific insight into the chemicals and antioxidant capacity of the ethanol/water (4:1) extracts of *E. valerianifolia* and *E. hieracifolia*. Besides, this study also reported the chemicals analysis including total phenolics (TPC) and total flavonoids (TFC) contents as well as Fourier transform infrared (FTIR). The ATR-IR data coupled with chemometrics discriminant analysis indicated the variability of chemicals in all samples.

MATERIALS AND METHODS

Sample Collection and Preparation

E. hieracifolia and *E. valerianifolia* leaves (biological replicates, n=6) were collected around Kuala Berang, Terengganu areas. The leaves were authenticated and voucher specimens were deposited to Faculty of Bioresources and Food Industry (FBIM) Herbarium, Universiti Sultan Zainal Abidin, Terengganu. The leaves were washed and dried before subjected to lyophilization. Briefly, the leaves were frozen in CHRIST flask at -20°C overnight and lyophilized by Freeze Dryer CHRIST Alpha 1-4Ldplus system to give in a powder form. Protocols for freeze dryer equipment; Main drying: -20 °C at 1.0 mBar, Final drying: -56 °C at 0.0018 mBar. Then, the leaves were ground to powder form.

Sample Extraction

The extraction method was carried out according to (Rashid et al. 2018).The sample (5 g) was macerated in 50 mL ethanol/water (4:1). The mixture was heated at 50°C for 15 minutes (min), sonicated for 15 min and centrifugated (4000 rpm, 15 min). The supernatant was concentrated (vacuo) and dried (under nitrogen gas flow) to crude extract. The crude extract was stored at 4 °C before being analyzed.

Antioxidant Assays

2,2-diphenyl-1-picrylhydracyl (DPPH) Radical Scavenging Assay

2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging assay was adopted according to Manzocco et al. (1998) with slight modification (Yunusa et al. 2019). Quercetin was used as standard. Test samples were prepared at concentrations in range of 500 to 3.9 µg/mL. 200 µL of DPPH reagent methanolic solution (0.125 mM in methanol) was mixed with 50 µL aliquots of sample. The mixture was incubated in a 96-well plate, under dark condition at room temperature (RT) for 30 min. The strength of the deep violet color in solution was measured at wavelength 517 nm using a microplate reader (Multiskan GO, Thermo Scientific, Finland). DPPH radicals reduction by antioxidants was reflected by the loss of deep violet color. The percentage inhibitions of DPPH radicals were calculated using the equation as given below:

$$\textbf{Inhibition (\%)} = [(A_o - A_1)/ A_o)] \times 100 \quad \text{… (Eq. 1)}$$

Where;
A_o = absorbance of the reagent control
A_1 = absorbance of the sample

2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) Scavenging Assay

2,2-azino-bis-(3-ethylbensthiazoline-6-sulfonic acid) (ABTS) scavenging was determined according to Wali and Alam (2019) with slight modification. Ascorbic acid was used as standard. Samples concentrations were in range of 500 to 3.9 µg/mL. $ABTS^{\bullet+}$ reagent solution was prepared by mixing 5 mg ABTS salt and 0.86 mg potassium persulfate in 20 mL distilled water (dH_2O) and stored for 12-16 hours (h) (under dark, RT) before

used. Then, the solution was diluted with methanol dropwise until the absorbance reached 0.700 at 734 nm. 50 µL of sample was mixed with 200 µL $ABTS^{\bullet+}$ reagent solution and incubated under dark condition (RT, 5 min). Then, the blue color reduction was read at wavelength 734 nm. The percentage of ABTS decolorization was calculated using Eq. 1.

Nitric Oxide (NO) Scavenging Assay

Nitric oxide was performed to determine the quantities of stable products of nitrate and nitrite (Amaeze et al. 2011). Quercetin was used as standard. The test samples were prepared at concentrations in range of 500 to 3.9 µg/mL. Griess reagent was prepared by mixing sulphanilic acid (0.33%), glacial acetic acid (20%) and naphthylethylenediamine dichloride (0.1%) in dH_2O. Sample (25 µL) was mixed with sodium nitroprusside (75 µL, 10 mM in PBS), and incubated in 96-well plate for 150 min. Then, 125 µL of Griess reagent was added. The mixture was incubated under dark (RT, 10 min). The absorbance of the pink color changes was measured at wavelength 546 nm using a microplate reader. The NO inhibition percentage was calculated using Eq. 1.

Ferric Reducing Antioxidant Power (FRAP) Assay

Ferric reducing antioxidant power (FRAP) assay was carried out based on the method described by Benzie and Devaki (2018) with some modification (Yunusa et al. 2019). The mechanism involves the reduction of the ferric-tripyridyltriazine (Fe^{3+}-TPTZ) by antioxidants samples to blue color ferrous-tripyridyltriazine (Fe^{2+}-TPTZ). FRAP working solution (colorless) was freshly prepared before used by mixing 300 mM acetate buffer, TPTZ (10 mM) in hydrochloric acid (40 mM) and iron chloride (20 mM) (10:1:1, v/v/v) and incubated in water bath (37°C, 10 min). Iron(II)sulphate ($FeSO_4.7H_2O$) at concentrations 125 to 1000 µM was used as standards to obtain the calibration curve. 15 µL test sample (100 µg/mL) or standard was mixed with 285 µL of FRAP solution and then incubated in water bath (37°C, 30 min). Then, the absorbance of Fe^{2+} concentration was read at wavelength 593 nm using a microplate reader. The results were expressed as millimoles of Fe^{2+} equivalents per gram of dried extract (mmol Fe^{2+}/g). The FRAP of samples was calculated based on following formula:

$$\mathbf{C = c \times V / m} \quad \ldots \text{(Eq. 2)}$$

Where;
c = concentration obtained from standard calibration curve (mg/mL)
V: volume of extract (mL)
m: weight of extract (g)

Total Phenolic Content (TPC)

Total phenolics content (TPC) assay was adopted from Ainsworth and Gillespie (2007) with slight modification (Rashid et al. 2019). The reaction was done in a 96-well plate. Gallic acid was used as a standard to obtain the calibration curve. Each aliquot of sample and gallic acid (20 µL) was added with 10% Follin Ciocalteau reagent (40 µL, v/v, dH_2O) and was vortexed (3 min) to avoid air oxidation of phenols and precipitation at bottom. Next, the mixture was added with sodium carbonate (160 µL, 7.5% w/v, dH_2O) to stop the reaction and was incubated (under dark, RT, 2 h). The blue color absorbance was measured at 765 nm using a microplate reader. The phenolic content of sample was calculated using Eq. 2. TPC was expressed as milligram of gallic acid equivalents per gram extract (mg GAE/g extract).

Total Flavonoid Content (TFC)

Total flavonoid content was carried out according to a modified protocol (Sembiring et al. 2018). Quercetin was used as standard. Samples and quercetin (60 µL, methanol) was added with aluminium chloride (30 µL, 10% w/v, dH_2O), 1 M potassium acetate (30 µL) and 52 µL dH_2O) and incubated at room temperature for 30 min. The yellow color changes were read absorbance at 415 nm using a microplate reader. The total flavonoid content of sample was calculated based Eq. 2. TFC was expressed as milligram of quercetin equivalent per gram extract (mg QE/g extract).

Attenuated Total Reflectance (ATR)-Fourier Transform Infra (FTIR) Spectroscopy

FTIR analysis of each biological replicate of extracts was performed using Perkin Elmer Spectrum 400 Infrared spectroscope equipped with air-cooled deutrated triglycin sulphate (DTGS) detector (Shimadzu, Nakagyo-ku, Kyoto, Japan). Sample of crude extract (10 mg) was applied directly on the platform crystal and tightened with the pressure clamp described by with minor modifications. The pressure clamp was placed at right above the samples and was tightened between the tip and opposing base. The spectra of absorbance mode were recorded in 16 interferograms (resolution of 4 cm^{-1}) at mid-infrared

region of 4000-400 cm^{-1}. Propylene glycol spectrum was used as a background. Each analysis was carried out in triplicate (Zin et al. 2019).

Statistical Analysis

Data was showed as mean ± standard deviation (n=6). One-way ANOVA and Tukey test was performed to evaluate significant differences between the extracts. Significant differences between samples were considered at $p<0.05$. FTIR spectra were smoothed and normalized with IR Solution version 1.40 (Shimadzu Corporation, Japan) and Spectragrypyh (Version 1.2) softwares. All spectra collected were analyzed using descriptive multivariate analysis of unsupervised pattern recognition including principal component analysis (PCA) and hierarchical cluster analysis (HCA) using MetaboAnalyst 5.0 online software.

RESULTS

Extraction

The extracts yield (w/w) of ethanol/water extracts (EW) of *E. hieraciifolia* (EH) and *E. valerianifolia* (EV) in range of 22.07% to 24.40% (w/w dry weight) were collected with no significant different between samples.

Antioxidant Activity

The antioxidant activity was determined using four antioxidant assays which were 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging, 2,2-azino-bis-(3-ethylbensthiazoline-6-sulfonic acid) (ABTS) radical cation, ferric reducing antioxidant power (FRAP) and nitric oxide (NO) scavenging assays. All the extracts depicted the tendency to scavenge the radicals in concentration-dependent pattern. The antioxidant activity of Erechtites sp. leaves extracts tested using four assays is presented in Figure 1 and Table 1.

2,2-Diphenyl-1-picrylhydracyl (DPPH) Free Radical Scavenging Activity

DPPH assay is a simple method used to evaluate the antioxidant activity of plant extracts. This assay can be used for both polar and nonpolar organic extracts to evaluate hydrophilic and lipophilic antioxidants (Prior et al. 2005). The method measures the inhibition capacity of the antioxidants in the extracts against DPPH radicals. The test samples DPPH radicals scavenging were found to be dose-dependent (Figure 1A). At concentration 500 µg/mL, EVEW inhibited stronger DPPH inhibition (84.14%) than EHEW (75.52%). The IC_{50} of EVEW against DPPH was 26.32 ± 3.98 µg/mL, while EHEW was 91.50 ± 31.81 µg/mL (Table 1).

2,2'-Azino-bis-(3-Ethylbenzothiazoline-6-sulphonic scid) (ABTS) Scavenging Activity

The ABTS radical cation assay is a simple and fast colorimetric method based on measurements of decaying $ABTS^{\bullet+}$ in the presence of antioxidant agents (Ilyasov et al. 2020). In this study, ABTS radical scavenging activity of the extracts increased in concentration-dependent manner, where at concentration 500 µg/mL, the scavenging activity was in range of 91.75-93.13% (Figure 1B). Based on the IC_{50} values, EVEW (20.42 ± 7.00 µg/mL) exhibited stronger ABTS scavenging activity compared to EHEW (33.07 ± 14.96 µg/mL) (Table 1).

Nitric Oxide (NO) Scavenging Activity

Quenching of nitric oxide (NO) radical is one of the methods to evaluate the antioxidant activity of the extracts. Nitric oxide interacts with oxygen, generated from reaction with sodium nitroprusside in aqueous solution to produce nitrites ions, then the quantities is measured by Griess Reagent (Pattananandecha et al. 2021). Both extracts at concentration 500 mg/mL did not inhibit up to 50% of NO radical (Figure 1C), thus IC_{50} of the extracts were calculated based on concentration 1000 mg/mL. The IC_{50} of EVEW was found to be significantly lower which indicated stronger antioxidant capacity than EHEW (Table 1) with respective values were 712.24 ± 58.24 and 938.93 ± 190.57 µg/mL.

Ferric Reducing Antioxidant Power (FRAP)

FRAP method evaluates the reduction of Fe^{3+}-tripyridyltriazine complex (Fe^{3+}-TPTZ) into a ferrous form (Fe^{2+}-TPTZ). High absorption at 593 nm indicates a stronger reducing power of the sample. Under acidic condition, an antioxidant compound reduce the ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}) in the form of TPTZ complex, producing a blue complex color (Fe^{2+}/TPTZ) (Benzie and Devaki, 2018). The current study showed that EVEW gave higher FRAP value at 18.56+1.98 mmol Fe^{2+}/g compared to EHEW with 12.83+3.58 mmol Fe^{2+}/g (Table 1).

Total Phenolic and Flavonoid Content

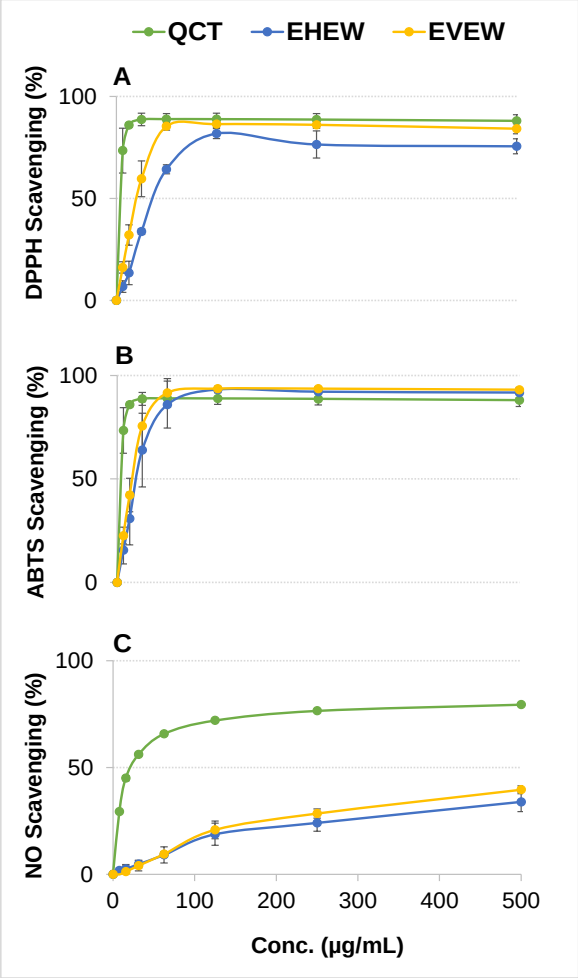
In this study, TPC values of EVEW (60.00 ± 5.82 GAE mg/g) was determined to be higher than EHEW (28.85 ± 10.31 GAE mg/g). Similar result was also found for TFC in which the values were

37.78 ± 5.22 and 23.70 ± 3.95 QE mg/g, respectively (Table 1).

ATR-FTIR Analysis of Erechtites sp.

FTIR Spectra

ATR-FTIR spectra of Erechtites sp. extracts attributed to stretching and bending vibrations of functional groups were coded in area 1 to area 5 (A1-A5) (Figure 2). The most variable characteristic of the spectra was determined in the fingerprint region of 1710-500 cm^{-1}.



Figures 1. Antioxidant activity of different concentrations of *Erechtites hieraciifolia* (EH) and *E. valerianifolia* (EV) extracts determined using different antioxidant assays (A-C). A) DPPH (%), B) ABTS (%), C) Nitric oxide (NO) (%) radicals scavenging activities. *Data represent mean ± standard deviation (n=6). EW = ethanol/water extract; QCT: Quercetin standard.

Table 1. Antioxidant capacity determined using DPPH, ABTS and NO radical scavenging activities, FRAP, TPC, TFC of extracts of *Erechtites hieraciifolia* (EH) and *E. valerianifolia* (EV).

Antioxidant Activity	Sample		
	EHEW	EVEW	QCT
DPPH (IC_{50}, µg/mL)	91.50 ± 31.81[a]	26.32 ± 3.98[b]	7.25 ± 0.72[c]
ABTS (IC_{50} µg/mL)	33.07 ± 14.96[a]	20.42 ± 7.00[b]	7.25 ± 0.72[c]
NO (IC_{50} µg/mL)	938.93 ± 190.57[b]	712.24 ± 58.24[a]	24.26 ± 0.82[c]
FRAP (mmol Fe^{2+}/g)	12.83 + 3.58[a]	18.56 + 1.98[b]	nd
TPC (GAE mg/g)	28.85 ± 10.31[a]	60.00 ± 5.82[b]	nd
TFC (QE mg/g)	23.70 ± 3.95[a]	37.78 ± 5.22[b]	nd

* Data represent mean ± standard deviation (n=6). Different alphabetical superscript indicates the significant different (p<0.05) among samples. QCT = Quercetin; IC_{50} = inhibition concentration at 50%; DPPH = 2,2-Diphenyl-1-picrylhydracyl; ABTS = 2,2′-Azino-bis-(3-Ethylbenzothiazoline-6-sulphonic acid); NO = nitric oxide, FRAP = Ferric-reducing antioxidant power; TPC = total phenolics content; TFC = total flavonoids content; GAE = Gallic acid equivalent; QE = Quercetin equivalent.

The spectra have broad peaks in area 1 and sharp peaks in area 4 and 5. The EVEW contained phytochemicals attributed to the strong peaks in area 2, 4 and 5, while phytochemicals in most EHEW triggered strong signals in area 2 and 4. The peaks in A1 (3600 to 3000 cm^{-1}) were assigned to stretching vibrations of hydroxyl group (O-H bonds) belonging to alcohols, phenolics, carbohydrates and peroxides components in celluloses, hemicelluloses and lignans. Signals in A2 (2900 to 2800 cm^{-1}) correspond to asymmetric (2916.37 cm^{-1}) and symmetric (2846.32 cm^{-1}) stretching of methylene (Crews et al. 2010). Meanwhile, bands in A3 (2500 to 1900 cm^{-1}) were assigned to the cumulated double bonds (C=C=C asymmetric) and triple bonds (C=C) stretching vibration. A4 (1800 to 1568 cm^{-1}) contais bands of C=O stretching

(carbonyl, ester and carboxylic acid), C=C stretching (aromatic skeletal) as well as N-H bending (amino acids) vibrations (Silverstein et al. 2014). Specifically, the C=O stretching vibrations of amide, lipids, and flavonoids was suggested for signals at 1685.36 cm^{-1}, while signals at 1661.68 cm^{-1} may belongs to C=C stretching vibrations of flavonoids (Lu et al., 2011) and C=O stretching of carbonyl of the carbohydrates (Kozłowicz et al. 2020). The fingerprints in A5 (1357 to 978 cm^{-1}) were presented as bending vibrations (O-H) and asymmetrical bending (C-CO) of hydrocarbon, as well as stretching vibrations of lipid and bending of tertiary alcohol groups. Stretching vibration (C-C) and bending (C-OH) of flavonoid and secondary alcohol group was assigned at 1111.71 cm^{-1}, elongation (C-C) and bending (C-OH) of primary alcohol group was assigned at 978.86 cm^{-1} (Easmin et al. 2017).

FTIR-Based Chemometrics

Principal Component Analysis (PCA)

FTIR spectra demonstrated complex data information attributed to different range of intensities and particular chemical functional groups. ATR-FTIR data sets of six biological replicates of the two Erechtites species were subjected to principal component analysis (PCA) for unsupervised classification to discriminate their chemicals characteristics.

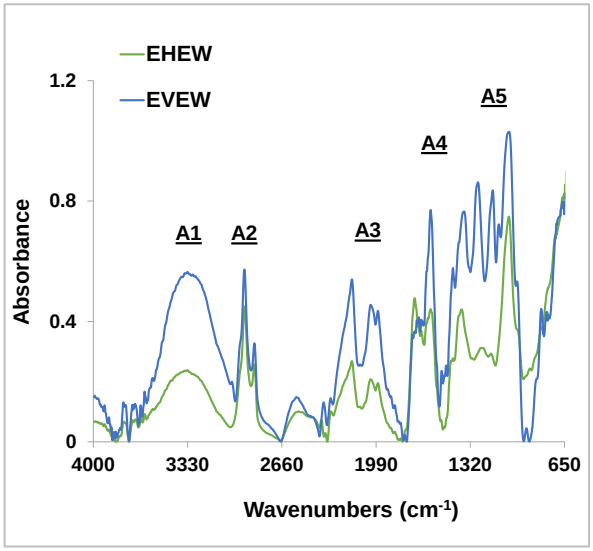


Figure 2. Representative Fourier transform infrared spectroscopy (FTIR) spectra of *Erechtites hieracifolia* and *E. valerianifolia* extracts.

The clustering in PCA model of FTIR spectra were observed, though some of the samples were overlapped (Figure 3). PCA dataset were analysed based on score plot and loading factor. The factor score discriminated the samples positions and showed a grouping of all samples in the first two PCA which explained 67.7% of the total variance. PC1 accounted for 43% and PC2 was 24.6%. The differences between samples were mostly observed in the absorbance range between 1800 to 1000 cm^{-1} as were known as the fingerprint region. EHEW2 to EHEW6 clustered together in PC1 demonstrated that they have similar chemical characters. Meanwhile, EVEW4 to EVEW6 clustered in a group in PC2. On the other hand, EHEW1, EVEW1, EVEW2 and EVEW3 were found to be farther from their respective groups.

Hierarchical Cluster Analysis (HCA)

Dendrogram from Hierarchical Cluster Analysis (HCA) of FTIR spectra of Erechtites species were shown in Figure 4. HCA is fast chemometric tools to discriminate all samples and was performed to observe the dissimilarities among the replicates. The relationships between species were calculated by Euclidean distance and Agglomeration (Ward's method) methods and displays in groups form.

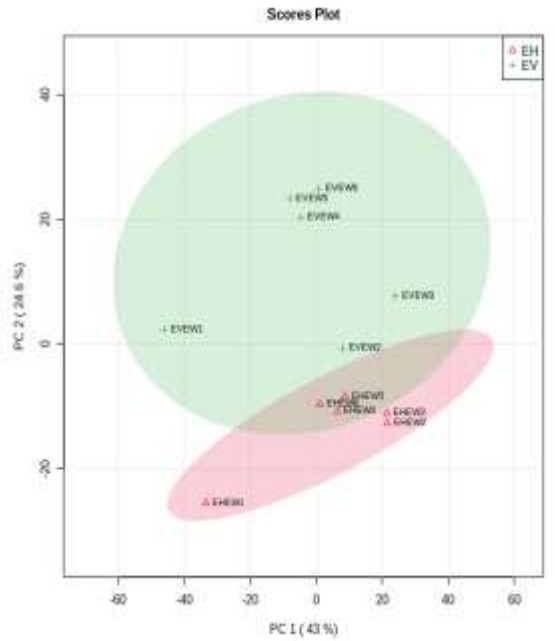


Figure 3. Principal components analysis (PCA) score plot of *Erechtites hieracifolia* and *Erechtites valerianifolia* ethanol/water (4:1) extracts.

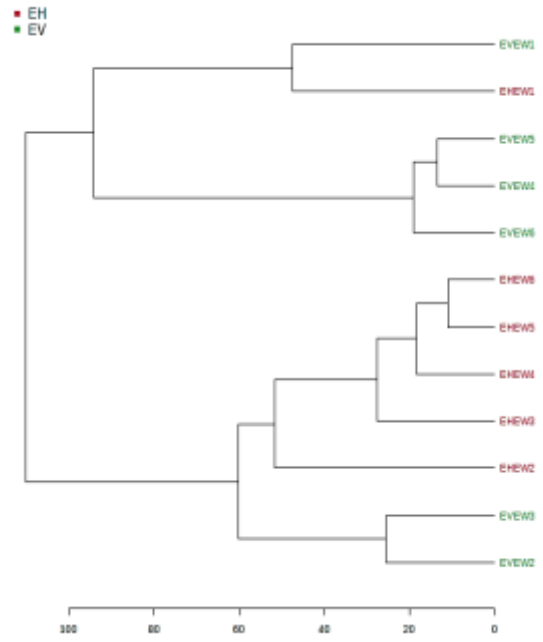


Figure 4: Hierarchical cluster analysis (HCA) dendrogram grouping of biological replicates of *Erechtites hieracifolia* and *Erechtites valerianifolia* ethanol/water (4:1) extracts.

HCA figure revealed samples grouping into three cluster; Cluster 1 (EHEW1, EVEW1), Cluster 2 (EVEW4-EVEW6) and Cluster 3 (EVEW2, EVEW3, EHEW2-EHEW6). These clustering showed clear variance of chemical characteristic of EHEW1 and EVEW1, thus revealing the samples as the outliers.

DISCUSSION

In this study, a freeze-drying method was used to avoid the degradation of the phytochemicals due to the sensitivity to high temperature. Freeze-drying known as lyophilization or cryodesiccation at low temperature is a dehydration process that comprises freezing of sample, lowering pressure, then sublimation. This technique prevents degradation of phytochemicals that often occur during drying of samples via heat treatment such as oven-drying (Mediani et al. 2012). Meanwhile, the ethanol/water (4:1) (EW) combination was selected as the extractant solvent due to the previous studies revealing that the ratio was optimal in retaining biologically active constituents such as phenolics (Mediani et al. 2015).

The antioxidant assays are classified into two mechanisms, namely single electron transfer (SET), and hydrogen atom transfer (HAT) methods. HAT method involves donation of a hydrogen ion from an antioxidant stable molecule to the free radical, thus reduced the reactive oxygen species (ROS), while SET method the antioxidant transfers an electron to the free radical thus reducing the oxidant or oxidizing agent (Shalaby and Shanab 2013). The half maximal inhibitory concentration (IC_{50}) was calculated to determine the concentration of extract needed to inhibit 50% of the oxidation function. Low IC_{50} values reflect a strong potency of antioxidant ability of the bioactive constituents indicating the potential for therapeutic purposes. In this study, four assays were tested including SET-based assay (DPPH and FRAP) and HAT-based methods (ABTS and nitric oxide) (Siddeeg et al. 2021), in order to provide a better understanding of mechanisms of action of the antioxidants in Erechtites sp..

The data revealed that *E. valerianifolia* exhibited lower IC_{50} in DPPH and ABTS assays as well as stronger FRAP compared to *E. hieraciifolia*, thus revealed that the antioxidants in *E. valerianifolia* extracts were effective to scavenge the ROS via both SET and HAT mechanisms. Comparing between the IC_{50} values, ABTS scavenging capacity of extracts were found to be stronger compared to DPPH scavenging, suggesting that stronger antioxidant reaction via HAT mechanism compared to SET. Besides, these findings also indicated that in some cases, the ABTS assay was more suitable to evaluate the hydrophilic antioxidants than DPPH due to ABTS solubility in both aqueous and organic solvents, thus enables better assessment of the antioxidant capacity of sample (Floegel et al. 2011).

EVEW also contained higher phenolics and flavonoids, which further showed *E. valerianifolia* as a potential source of antioxidants including the phenolics. The phenolics have been extensively reported in medicinal plants and the high amount of TPC in the extract has been commonly corresponding to the stronger antioxidant activity (Rashid et al. 2019). The presence of phenolics including flavonoids were proven with detection of FTIR bands at 1514.84 cm^{-1} (A4) which attributed to stretching vibrations aromatic C=C ring of flavonoids. Besides, the symmetrical bending vibration of C-H bonds and C-C aromatic skeletal of phenyl groups occurs at 1373.21 cm^{-1} and C-O stretching vibrations of phenols or tertiary alcohol at 1117 cm^{-1} were also detected (Thummajitsakul et al. 2020). In additions, the peaks at 1048.38 cm^{-1} and 978.85 cm^{-1} were assigned to ribose C-O stretching, primary alcohol and C-O stretching

vibrations secondary alcohols. The carbohydrate signals were also assigned approximately at 1400-900 cm^{-1} (Rashid et al. 2009). The presence of fingerprints of lipids, proteins, polysaccharides and polyphenols ranging from 1800-800 cm^{-1} was previously discussed (Lu et al. 2011).

The chemometrics analysis of infrared spectra involving PCA and HCA showed variance of chemical characteristic of *E. valerianifolia* and *E. hieraciifolia*. PCA loading plot give a better understanding of discrimination of samples since the dataset revealed the fingerprints which contributed to the variability. The strong loading (>0.75) variables of PC1 and PC2 were found in peaks of A1 assigned to C-O stretching vibration of methyl and methylene group. The PCA and HCA showed that among the biological replicates the samples EHEW1 and EVEW1 could be considered as the outliers due to their clustering farther from other samples. Meanwhile, PC2 cluster were influenced by strong loading variable in A5 which mainly contain varieties of phenols and flavonoids fingerprints.

CONCLUSION

These findings provide better understanding of the chemicals variability as well antioxidant potential of both Erectites sp. leaves extracts which are common edible vegetables for local people. *E. valerianifolia* leaves extracted with 80% ethanol were significantly proven to exhibit stronger antioxidant activity compared to *E. hieracifolia*. The results indicated *E. valerianifolia* (EV) has potential as a promising source of antioxidant against free radicals as the leaves possess high amounts of phenolics and flavonoids as well as strong antioxidant activities. In general, this study has contributed towards further promoting the researches on both species. Specific further study on the chemical characterization of the antioxidants was required to discuss further on the structural variability-activity relationship since outliers were detected among samples.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

NA designed and performed the experiments and also wrote the manuscript. BMZ helped in analyzing the data. ZMR designed experiments, edited and reviewed the manuscript. All authors read and approved the final version.

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