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A Comparative Study of Various Extraction Techniques for Extracting Antioxidant-Rich Phytoconstituents from *Eryngium foetidum* Leaves Using Spectrophotometric and HPLC Methods

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Abstract: *Eryngium foetidum* L. (Apiaceae) is known to possess numerous healthcare properties and has been utilized in the traditional system of medicine for various health issues. However, scientific data on its phytochemistry and antioxidant properties is limited. Therefore, this study aimed to document the phytochemistry and antioxidant properties of leaves by employing different extraction techniques to obtain plant constituents. Sonication (EN1), Soxhlet (EN2), maceration (EN3), and maceration with heat (EN4) were used as the extraction techniques while water was used as the extracting solvent. HPLC method associated with a PDA detector was developed to compare the phytochemicals profile of *E.foetidium* under different extraction techniques. The antioxidant capacities and the content of saponins (SC), terpenoids (TC), flavonoids (TFC), tannins (TTC), alkaloids (AC), and polyphenolics (TPC) were determined spectrophotometrically. The extraction techniques EN2 and EN4 were identified as yielding the highest overall results and giving a wide range of phytochemicals in the HPLC-PDA method. The quantitative analyses resulted in high SC, TTC, TC, and TPC in the EN4 (185.84±0.54 mg SE/g, 36.99±0.64 mg TAE/g, 0.89±0.01 mM LE/g, and 37.37±0.65 mg GAE/g, respectively) and low in the EN1 extraction techniques. TFC levels in EN2 were high (11.84±0.14 mg QE/g), whereas it was low in EN3. Furthermore, AC was higher in the extraction method EN3 (1.67±0.01 mg AE/g) and lower in the extraction technique EN2. The total antioxidant capacity was higher in the EN4 extract (47.17±0.20 mg Trolox Eq/g) and lower in the EN1 extract. The lowest IC_{50} in the 2,2′-Diphenyl-1-Picrylhydrazyl (DPPH) assay was noted for EN3 extract (12.91±0.02 mg/mL) revealing the highest scavenging activity than the other extracts. Based on HPLC and spectrophotometric applications, maceration with heat (EN4) is recommended for efficiently extracting polyphenols and antioxidants from *E. foetidum* leaves. The application of heat would also improve the extraction efficiency of phytochemicals.

Keywords: *Antioxidants, extraction methods, Eryngium foetidum, HPLC analysis, phytochemicals, spectrophotometric analysis.*

1. Introduction

Eryngium foetidum L. (Apiaceae), long coriander, is a biennial, pungently scented tropical plant that is also known as spiny coriander and Andu in Sinhala (Dalukdeniya & Rathnayaka, 2017; Dawilai, Muangnoi et al., 2013; Garcia et al., 1999; Okon et al., 2013). *E. foetidum* is found in the Kingdom Plantae, Division Tracheophyta, Class Magnoliopsida, Order Apiales, Family Apiaceae, Genus *Eryngium*, and Species *foetidum* (Dalukdeniya & Rathnayaka, 2017). *E. foetidum* is utilized as a healthy food due to its high levels of vitamins, riboflavin, iron, calcium, carotene, and proteins. This plant is utilized in traditional medicine to cure a variety of illnesses, including malaria, hypertension, fevers, vomiting, chills, headaches, asthma, burns, earaches, snake bites, stomachaches, scorpion stings, diarrhea, epilepsy, and arthritis (Dawilai et al., 2013; Eyoum Bille & Nguepi, 2016; Promkum et al., 2012). Because of its strong fragrance, its leaves are utilized as a flavoring in many dishes (Dawilai et al., 2013). *E. foetidum* is also used as a culinary spice and is frequently used in the fragrance and cosmetic industries (Okon et al., 2013). This plant has been shown to have anti-inflammatory, anthelmintic, anticonvulsant, analgesic, anticarcinogenic, anticlastogenic, antibacterial, and antidiabetic action due to the availability of important phytoconstituents, including alkaloids, polyphenolics, flavonoids, saponins, tannins, and terpenoids (Eyoum Bille & Nguepi, 2016; Promkum et al., 2012). Even though *E. foetidum* leaves contain important bioactive molecules, the quality and quantity of phytochemicals extracted depend on the solvents and extraction techniques employed (Anusha et al., 2013; Chandira & Jaykar, 2013; Eyoum Bille & Nguepi, 2016; Lingaraju et al., 2016; Malik et al., 2016; Okon et al., 2013).

The selection of a proper extraction technique is crucial due to the highly complex nature of phytochemical composition and the presence of trace amounts of some phytochemicals. (Cannel, 1998). The extraction methods, namely, maceration, Soxhlet extraction, decoction, percolation, digestion, counter-current extraction, infusion, fermentation, ultrasound-assisted, supercritical fluid, microwave-assisted, distillation methods, etc., are widely used in the extraction of natural products (Abubakar & Haque, 2020; Devgun et al., 2010; Handa et al., 2008; Hanif et al., 2019; Manousi et al., 2019; Mtewa et al., 2018; Pandey & Tripathi, 2014; Stratakos & Koidis, 2016). The extraction method used determines the accuracy and precision of both quantitative and qualitative measurements of plant-based phytoconstituents, as

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well as the stability of phytochemical components (Azwanida, 2015; Li et al., 2007). Therefore, developing effective and targeted extraction and isolation processes for bioactive natural compounds is required. Chemical profiling using HPLC and phytochemical quantification through spectrophotometric methods must be accurate and efficient methods for comparing different extracts applying varying extraction techniques and selecting the best method to extract antioxidant-rich fractions from *E. foetidum* leaves.

Now, the world's current tendency is to develop healthy, value-added products such as functional foods and nutraceuticals for the prevention of many non-communicable diseases. Therefore, the food industry and natural product researchers would benefit from the outcome of this study. Few research reports are available on the extraction of phytochemicals from *E. foetidum* leaves using water as the extracting solvent (Chandira & Jaykar, 2013; Lingaraju et al., 2016; Malik et al., 2016). Therefore, the objective of the present study was to establish appropriate extraction techniques for extracting highly essential phytoconstituents such as flavonoids, alkaloids, tannins, saponins, terpenoids, polyphenolics, and antioxidant-rich fractions from *E. foetidum* leaves applying HPLC techniques and spectrometric methods and to fill the gap of research literature on *E. foetidum*.

2. Materials and Methodology

Plant Materials and Chemicals

E. foetidum leaves were obtained in Tangalle, Matara, Sri Lanka (longitude 80.7856 ºE and latitude 6.0289 ºN). The specimen was authenticated and deposited (voucher No. AHEAD/DOR 05/C1) in the National Herbarium, Peradeniya, Sri Lanka.

Absolute ethanol (EtOH), acetic anhydride, aluminum chloride anhydrous ($AlCl_3$), ammonium hydroxide (NH_4OH), aromocresol green (BCG), n-butanol, chloroform ($CHCl_3$), copper acetate, copper sulfate ($CuSO_4$), 2,2'-diphenyl-1-picrylhydrazyl (DPPH), dimethyl sulfoxide (DMSO), ferric chloride hexahydrate ($FeCl_3{\cdot}6H_2O$), folin-Ciocalteu reagent (FC reagent), glacial acetic acid (CH_3COOH), gallic acid monohydrate, hydrochloric acid (HCl), lead acetate, linalool, magnesium ribbon (Mg), nitric acid (HNO_3), phosphomolybdic acid, potassium hydroxide (KOH), pyridine, olive oil, sodium chloride (NaCl), sodium carbonate monohydrate ($Na_2CO_3{\cdot}H_2O$), sodium hydroxide (NaOH), sodium nitroprusside, sulfuric acid (H_2SO_4), tannic acid, trolox, and 2,4,6-tripyridyl-s-triazine are classified as AR grade chemicals, benzene, diethyl ether, methanol, and hexane are come under GC grade chemicals and quercetin is comes under HPLC grade. All the chemicals were purchased from Merck and Sigma Aldrich.

Phytochemical Extraction

The collected leaves from *E. foetidum* were dried under shade in a ventilated room for about 24 hours to eliminate moisture from the surface of the washed leaves. A grinder (HL 7756 09) was then used to grind the sample. Four different extraction strategies were employed to select the best method: sonication (EN1, 1 hr, room temperature, 40 kHz), Soxhlet (EN2, 105 ºC, 6 hrs), maceration with agitation (EN3, room temperature, 6 hrs, 1000 rpm), and maceration with agitation upon heating (EN4, 60 ºC, 6 hrs, 1000 rpm) (Kokilananthan, Vajira, Gangabadage, & Harshi, 2022). About 100.00 g of *E. foetidum* leaves and 500.0 mL of distilled water were utilized in all extraction techniques. To ensure the reproducibility of the extraction yields, all four extraction processes were tripled. Cotton plugs and Whatman (No. 1) filter paper was utilized to filter the extracts. At 50 °C, the filtrates were concentrated with a rotary evaporator (Model No: HS-2005S), and the moisture was removed with a freeze dryer (S/No: FD 2020062222, Model: FE-10-MR) (Mtewa et al., 2018). The crude powder obtained was stored at -30 ºC until further application.

Samples Preparation for HPLC Applications

Chemical profiling/ identification with analytical HPLC was used to compare all four aqueous extraction techniques mentioned in this study (Al-Rimawi et al., 2017; Al-Rimawi et al., 2018; Sathyanarayanan et al., 2017). Impurities like nonpolar compounds were removed by, re-extracting the *E. foetidum* leaf aqueous extracts with diethyl ether and dichloromethane (tripled). The water layers of the extracts were concentrated using a rotary vacuum evaporator and then subjected to freeze-drying. For the HPLC applications, 500 ppm solutions of all four of them were prepared.

HPLC-PDA Application for Chemical Profiling of All Four Different Extracts

HPLC analyses were performed with a SHIMADZU LC-20AP liquid chromatograph (Japan) with four solvent delivery system quaternary pumps (FCV-200AL), including a photodiode array detector (SPD-M40). The SHIMADZU LC was comprised of a degasser (DGU-10B), and the analytical line was especially interconnected with an autosampler (SIL-10AP), and column oven (CTO-20AC). All of these modules were linked to the communication bus module (CBM-20A), which was then linked to the computer system, which was running LabSolutions (SHIMADZU) software as a data processor. The compound identification was analyzed by analytical HPLC technique with the analytical column: Shim-pack GIST C18-AQ µm, 4.6 I.D.×150 mm. The analytical method was developed by changing the solvents' polarity and flow rate. Ultra-pure distilled water and methanol were used as the solvents (a solvents system was developed with the help of TLC analysis). After the method was developed with several runs, samples were run with the developed method as follows:

The autosampler injected about 10.0 µL of sample into the column. A gradient solvent system made of ultra-pure water and methanol was used to elute the samples through the column at a flow rate of 1 mL/min. Before injecting into the column, the solvent gradient was set to begin with pure water and end with pure methanol. The samples were monitored by the PDA director with wavelengths ranging from 190 to 800 nm at 35 ºC of column oven temperature.

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Phytochemical Qualitative Analysis

Using established techniques outlined in the literature (Abubakar & Haque, 2020; Gayathri & Kiruba, 2014; Kokilananthan et al., 2022b; Wadood et al., 2013), bioactive substances such as alkaloids, flavonoids, terpenoids, saponins, polyphenolics, tannins, glycosides, coumarin, anthocyanins, phytosterols, quinones, chalcones, and betacyanin were qualitatively tested in all aqueous extracts of *E. foetidum* leaves. Detailed procedures are shown in Table 1.

Table 1: Qualitative tests for phytochemical screening

Phyto- chemicals	Test method	Procedure	Observation for indicating a positive test	Reference
Alkaloids	Mayer's Test	1.0 mL plant extract (1% HCl used for the extraction) + 1.0 mL Mayer's reagent	Creamy white or yellow precipitate	(Banu & Cathrine, 2015)
	Wagner's Test	1.0 mL plant extract (1% HCl used for the extraction) + 1.0 mL Wagner's reagent	A brown or reddish precipitate	
	Dragendroff's Test	1.0 mL plant extract (1% HCl used for the extraction) + 1.0 mL Dragendroff's reagent	Reddish-brown or orange-red precipitate	
Glycosides	Keller-kilani Test	5.0 mL plant extract + 2.0 mL glacial acetic acid + 5% $FeCl_3$ solution (few drops) + 1.0 mL Con. H_2SO_4	A brown ring at the interface or violet ring may emerge under the brown ring, and a greenish ring may grow gradually across the acetic acid layer.	(Biswas, Rogers, McLaughlin, Daniels, & Yadav, 2013; Sawant & Godghate, 2013)
	Modified Borntrager's Test	5.0 mL plant extract+ 5% $FeCl_3$ solution (few drops) → keep mixture in a boiling water bath for 5 min → Extract with benzene → 1% NH_4OH react with benzene layer	Rose-pink color formation	
	Legal's Test	2.0 mL plant extract + 0.5% sodium nitroprusside (1.0 mL) + 2.0 mL pyridine + 2.0 mL NH_4OH (10%)	Pink to blood-red coloration	
Flavonoids	Alkaline reagent Test	5.0 mL plant extract + few drops NaOH (10%) (+ few drops dil. H_2SO_4)	Bright yellow coloration becomes colorless with the addition of acid	(Arya, Thakur, & Kashyap, 2012; Sawant & Godghate, 2013; Shaikh & Patil, 2020; Sheel, Nisha, & Kumar, 2014; Wadood et al., 2013)
	Shinoda Test/ Mg turning Test	2.0 mL plant extract + metal magnesium (0.50 g) + few drops Con. HCl	Pink to red color formation	
	Lead acetate Test	2.0 mL of extract + a few drops of lead acetate (5%)	White or yellow precipitate	
	$AlCl_3$ Test	3.0 mL plant extract + 4.0 mL $AlCl_3$ solution (1%)	Yellow precipitate	
	NH_4OH Test	3.0 mL plant extract + 5.0 mL NH_4OH solution + 1.0 mL Con. H_2S0_4 solution	Yellow color formation	
Saponins	Froth Test	1.0 mL plant extract + 5.0 mL distilled water (Shake well)	The foam formed lasts for 10 minutes	(Biswas et al., 2013; Mohlakoana & Moteetee, 2021)
	Olive Oil Test	10.0 mL plant extract (aqueous) + few drops olive oil → shake well	Creation of an emulsion	

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Tannins	Braymer's Test	2.0 mL plant extract + 2.0 mL $FeCl_3$ (10%)	Appearance of deep greenish-grey or blue-black color	(Savithramma et al., 2011; Venkateswarlu et al., 2014)
	Lead Acetate Test	1.0 mL plant extract + 3 drops lead acetate solution	A creamy gelatinous precipitate	
Terpenoids	Salkowski's Test	1.0 mL plant + 2.0 mL chloroform +2.0 mL Con. H_2SO_4	Golden yellow or reddish-brown in the interphase	(Gayathri & Kiruba, 2014; Sawant & Godghate, 2013)
	Liebermann-Burchardt Test	1.0 mL plant extract + few drops acetic anhydride → boil & cool → Con. H_2SO_4 was added from the wall of the test tube	Brown ring at the interphase of two layers	
	Copper acetate Test	2.0 mL plant extract (aqueous) + a few drops of copper acetate solution (5%)	Development of the emerald-green color	
Poly- phenolics	Ferric Chloride Test	2.0 mL plant extract + few drops alcoholic $FeCl_3$ solution	Development of a bluish-black	(Sawant & Godghate, 2013)
Coumarins	UV light Test	1.0 mL plant extract in test tube → Test tube mouth is covered with NaOH (1N) treated filter paper → heated for a few minutes in a water bath	Yellow fluorescence in the paper under UV light	(Sawant & Godghate, 2013; Rajesh et al., 2014)
	NaOH Test	2.0 mL plant extract + 3.0 mL NaOH (10%)	Formation of yellow color	
Anthocyanins	HCl & NH3 Test	2.0 mL plant extract + 2.0 mL HCl (2 N) + 2.0 mL NH_4OH solution	Development of pink-red to blue-violet coloration	(Sawant & Godghate, 2013)
Chalcones	NaOH Test	2.0 mL plant extract + 2.0 mL NH_4OH solution	Development of the red color	(Sawant & Godghate, 2013)
Phytosterol	Salkowski's Test	2.0 mL plant extract + 2.0 mL chloroform + few drops Con. $H2_SO_4$	Development of golden red	(Sawant & Godghate, 2013)
Betacyanin	NaOH Test	2.0 mL plant extract + 1.0 mL NaOH (2 N) → heated for 5 minutes at 100 ºC	Development of yellow color	(Rajesh et al., 2014)
Quinones	H_2SO_4 Test	2.0 mL plant extract + 1.0 mL Con. $H2_SO_4$	Development of a red color	(Rajesh et al., 2014)

Phytochemical Quantitative Analysis

Each 0.10 g aqueous extract of *E. foetidum* leaves was dissolved in 0.25 mL of DMSO and diluted with 100.0 mL of MeOH to obtain a 1000 ppm concentration solution for phytochemical spectrophotometric analyses. Phytoconstituents such as terpenoids, polyphenolics, alkaloids, flavonoids, tannins, and saponins were determined based on the methods described in the literature (Kokilananthan et al., 2022a; Kokilananthan et al., 2021; Kokilananthan et al., 2020; Kokilananthan et al., 2022b; Shanthirasekaram et al., 2021).

The Folin-Ciocalteu reagent technique was employed to measure the tannin content (TTC) and phenolic content (TPC). In summary, 0.5 mL of the developed sample extract was mixed with 2.5 mL of the FC reagent mixture and permitted to stand for 5 minutes. After that, 2.0 mL of Na_2CO_3 (7.5% w/v) solution was introduced and incubated for 30 minutes. The absorbance was measured at 765 nm. For TTC, tannic acid was utilized as the standard, and the outcomes were measured in mg TAE/g, whereas for TPC, gallic acid was utilized as the standard, and the findings were presented in mg GAE/g.

To evaluate the amount of flavonoids in a sample (TFC), a spectrophotometric approach with a working solution of $AlCl_3$ was employed. In summary, 1.0 mL of sample extract was treated with 0.5 mL of $AlCl_3$ (2%) working solution and 0.5 mL of distilled water and allowed to stand for 10 minutes before detecting absorbance at 425 nm. Quercetin was used as a control, and the findings were reported in mg QE/g.

The terpenoid content (TC) was examined using a spectrophotometric approach with a phosphomolybdic acid working solution. To summarize, 1.0 mL of aqueous phosphomolybdic acid solution (5%) was gradually added to 1.0

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mL of sample extract, followed by 1.0 mL of con. H_2SO_4. The mixture was thoroughly mixed and allowed to stand for 30 minutes before being diluted with MeOH to 5.0 mL. At 700 nm, the absorbance was measured. Linalool was used as the standard, and the findings are presented in mM LE/g.

A spectrophotometric method with a vanillin-sulfuric acid working solution was utilized to determine saponin content (SC). Simply put, 8% vanillin (1.0 mL) was mixed with an equal quantity of sample extract before it was placed in an ice bath, followed by 8.0 mL of H_2SO_4 (77%). The test tube was shaken before being placed in a 60 °C oven for 30 minutes. At 540 nm, the absorbance of the solution was measured after it had reached room temperature. The findings were presented in mg SE/g, and saponin was utilized as the standard.

Spectrophotometric analysis was used to determine the alkaloid content (AC) of the samples using a bromocresol green working solution. The plant extract was dissolved in a solution of 2M HCl. 10.0 mL of chloroform was used to rinse 1.0 mL of this supernatant. 0.1M NaOH was used to bring the pH of this produced sample to neutral. This was then combined with freshly prepared BCG solution (5.0 mL) and phosphate buffer solution (pH 4.7, 5.0 mL). The complex mixture was re-extracted with chloroform (1.0, 2.0, 3.0, and 4.0 mL). The extracted complex mixture was adjusted to 10.0 mL using chloroform. At 470 nm, the complex's absorbance was determined. The findings were presented in mg AE/g and measured using atropine as the standard.

Antioxidant Analysis

To test the radical scavenging capability of all aqueous extracts of *E. foetidum* leaves, the DPPH radical scavenging assay, a standardized existing approach published in the literature, was used (Abeysuriya et al., 2021; Blois, 1958; Brand-Williams et al., 1995; Kokilananthan et al., 2021; Kokilananthan et al., 2020). About 100 µL of aqueous extract (in different concentrations) was mixed with the 3.9 µL of 0.06 mM DPPH working solution. The absorbance at 517 nm was measured after 30 minutes in complete darkness. The IC_{50} value for free radical scavenging activity was assessed by a plot of the scavenging effect's percentage versus concentration. , Trolox and ascorbic acid were used as standards. A standard method that has been described in the literature was employed to measure the FRAP value of all obtained extracts from the leaves of *E. foetidum* (Biglari et al., 2008; Firuzi et al., 2005; Gliszczyńska-Świgło, 2006; Kokilananthan et al., 2021; Kokilananthan et al., 2020). The 100 µL test sample was combined with 3.0 mL of freshly prepared FRAP solution. The absorbance at 593 nm was measured after 30 minutes of incubation at 37 °C. The standard Trolox was used for the calibration.

Statistical Analysis

The data was analyzed and compared using Cochran's Q-test (non-parametric statistics) and the T-test (LSD). Statistical analysis were conducted using, R-studio software and SAS OnDemand for Academics: Studio (SAS 9.4). Using means and standard deviations, the data were displayed.

3. Results and Discussion

Extraction of Phytochemical

The yield percentages of all four different aqueous extracts of *E. foetidum* leaves were compared and statistically analyzed; the results are shown in Figures 1 and 2. of the four different extraction techniques (EN1, EN2, EN3, and EN4), the technique EN2 (5.73 ± 0.07%) produced the highest yield, followed by EN4 (5.47 ± 0.06%), EN3 (3.45 ± 0.08%), and EN1 (2.57 ± 0.09%). It is worthy of note that, as shown in Figure 2, statistical analysis data for all four *E. foetidum* leaf extracts revealed that four extraction methods generated different proportions at a 5% significant level. Even though many previous studies have been conducted using different extraction techniques and extracting solvents, most of the reports have not included the yield percentage to compare the results with current findings (Anusha et al., 2013; Eyoum Bille & Nguepi, 2016; Okon et al., 2013).

When comparing the extraction techniques EN3 and EN4, both were carried out under the same circumstances except for temperature; EN3 was carried out at room temperature, while EN4 was carried out at 60 ºC. As a result, there is a significant assertion for extraction yield variations with extraction techniques EN3 and EN4. This implies that the temperature had an impact on the extraction yield, as EN4 had a larger extraction yield than EN3. Although EN1, one of the most sophisticated extraction techniques (Mtewa et al., 2018), was used, its yield percentage was lower than that of the other techniques. This must be due to the extraction period, which is one hour in EN1 at room temperature. As a result, further studies are needed to optimize the extraction parameters, particularly the temperature and time.

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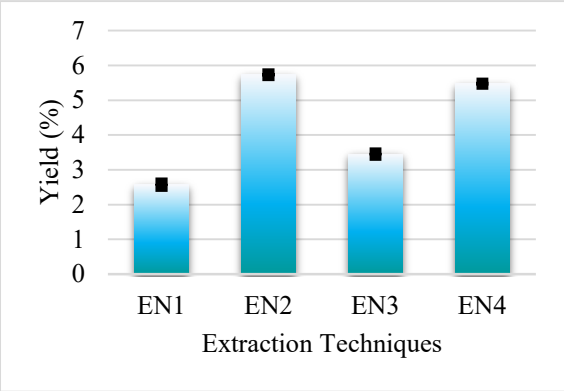

Figure 1: Yield percentage of four different extraction approaches utilized for extracting phytochemicals from *E. foetidum*.

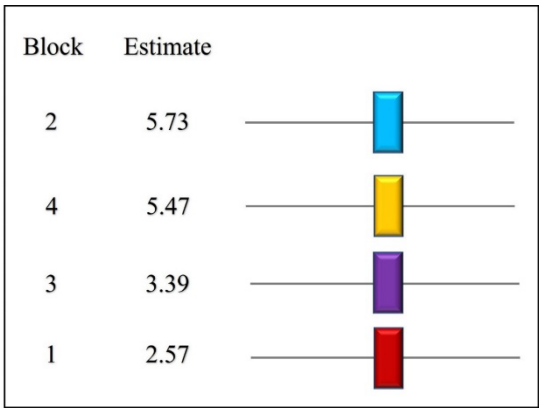

Figure 2: Statistical proof comparing the extraction yields of the four different extraction methods used to extract the phytoconstituents from *E. foetidum* leaves (Block: 1: EN1, 2: EN2, 3: EN3, 4: EN4, Alpha = 0.05, Estimate: the mean values covered by the same bar do not differ significantly.).

HPLC-PDA Chemical Profiling of All Four Different Aqueous Extracts

The HPLC technique was utilized to compare four various aqueous extracts of *E. foetidum* leaves to determine which extraction method would extract most of the phytochemicals from the plant source. As a result, an HPLC method for *E. foetidum* aqueous extracts was established, and the results were compared in various ways. According to the maximum wavelengths of the identified compounds, the extracted substances in all four extracts were first directly examined on HPLC-PDA at different wavelengths, including 254 nm, 204 nm, 654 nm, 734 nm, and 224 nm. The maximum wavelengths of most of the identified substances fall within 654 nm. Figure 3 shows the HPLC-PDA spectra from all four aqueous extracts at 654 nm.

As shown in Table 2, EN2 and EN4 extracts have more peaks or compounds in the HPLC-PDA spectra for all five different wavelengths than the other two extraction techniques. To , only the peaks with areas greater than 5×10^6 counts and heights greater than 4×10^5 counts in the HPLC-PDA spectra at all five different wavelengths were considered. As shown in Table 3, more of the higher-intensity peaks were also found in the extracts obtained by the EN2 and EN4 extraction methods. All the peaks identified in these conditions were studied and tabulated in Table 3 to be more selective.

Table 2: HPLC-PDA spectra peak data with different applications

Methods	**Extraction technique and total no of the peaks**			
	EN1	EN2	EN3	EN4
Wavelength 254 nm, 4nm	152	231	198	215
Wavelength 204 nm, 4nm	168	228	188	233
Wavelength 654 nm, 4nm	154	224	196	212
Wavelength 734 nm, 4nm	154	238	200	212
Wavelength 224 nm, 4nm	155	237	197	215

The summarized data are tabulated in Table 3 based on the peak area and peak height. Table 3 demonstrates that the extraction technique has an impact on the extraction of a single compound. Simply put, all extracts at 254 nm had a single peak at that wavelength, as shown in Table 3, except for EN1, for which there was none. Remarkably, the RT of the observed peaks differs depending on the extraction technique. That indicates that each extraction method is specific to the compounds of interest. Conversely, the same compounds were also found in all four extracts at varying levels of intensity. These findings demonstrate how important the selection extraction technique is in the extraction of desired substances from natural sources.

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Table 3: HPLC-PDA spectra peaks data which greater than the area of 5×10^6 counts and peaks data which is greater than the height of 4×10^5 counts

Methods	Functions	Total Peaks	Peak No	Extraction Techniques							
				EN1		EN2		EN3		EN4	
				RT	RP	RT	RP	RT	RP	RT	RP
204 nm, 4nm	Area	4	1	5.32	92.76	4.33	40.94	5.56	30.28	6.19	33.38
			2	77.40	4.06	31.02	12.25	28.11	17.95	42.00	6.93
			3	99.66	3.18	77.61	13.43	77.45	17.30	77.42	17.45
			4	……	……	99.68	33.38	99.66	34.47	99.65	42.25
	Height	5	1	5.32	61.59	4.33	35.56	5.55	39.47	6.18	39.48
			2	31.13	38.41	26.36	16.02	26.30	18.45	31.39	15.7
			3	……	……	31.02	16.51	28.11	42.08	33.69	14.38
			4	……	……	31.15	14.79	……	……	35.00	14.29
			5	……	……	35.92	17.12	……	……	99.65	16.14
224 nm, 4nm	Area	3	1	5.34	100.00	4.34	61.58	5.59	44.41	6.20	38.40
			2	……	……	31.02	38.42	28.11	55.59	42.00	23.96
			3	……	……	……	……	……	……	99.65	37.65
	Height	4	1	31.13	57.23	26.36	25.46	26.30	30.08	6.20	23.88
			2	39.41	42.77	31.02	25.66	28.11	69.92	31.39	27.52
			3	……	……	31.15	22.68	……	……	33.69	23.89
			4	……	……	35.92	26.21	……	……	35.00	24.72
254 nm, 4nm	Area	1	1	……	……	31.02	100.00	28.11	100.00	42.00	100.00
	Height	6	1	31.13	55.39	26.36	18.19	26.30	22.91	27.65	23.49
			2	39.41	44.61	31.02	17.7	28.11	54.19	31.39	27.1
			3	……	……	31.15	15.47	39.45	22.9	32.88	24.27
			4	……	……	35.17	15.53	……	……	35.00	25.14
			5	……	……	35.92	18.09	……	……	……	……
			6	……	……	50.95	15.02	……	……	……	……
654 nm, 4nm	Area	3	1	39.41	100.00	31.01	100.00	28.13	100.00	31.39	34.81
			2	……	……	……	……	……	……	42.00	33.46
			3	……	……	……	……	……	……	68.20	31.73
	Height	6	1	31.13	36.55	26.36	18.72	23.78	14.72	27.65	19.62
			2	39.41	34.97	31.01	17.39	26.30	14.44	31.39	21.71
			3	48.04	28.48	31.15	14.62	28.13	38.82	32.88	20.82
			4	……	……	35.17	16.27	39.45	16.58	35.00	20.69
			5	……	……	35.92	17.69	44.42	15.43	36.61	17.16
			6	……	……	50.95	15.31	……	……	……	……
734 nm, 4nm	Area	3	1	39.41	100.00	31.01	100.00	28.13	100.00	31.39	34.32
			2	……	……	……	……	……	……	42.00	32.52
			3	……	……	……	……	……	……	68.20	33.16
	Height	6	1	25.37	28.28	26.36	18.57	23.78	14.73	27.65	19.58
			2	31.13	37.09	31.01	17.64	26.30	14.79	31.39	21.99
			3	39.41	34.63	31.15	14.63	28.13	38.64	32.88	20.74
			4	……	……	35.17	15.75	39.45	16.42	35.00	20.44
			5	……	……	35.92	17.93	44.42	15.42	36.61	17.26
			6	……	……	50.95	15.49	……	……	……	……

RT: Retention time, RP: Relative percentage

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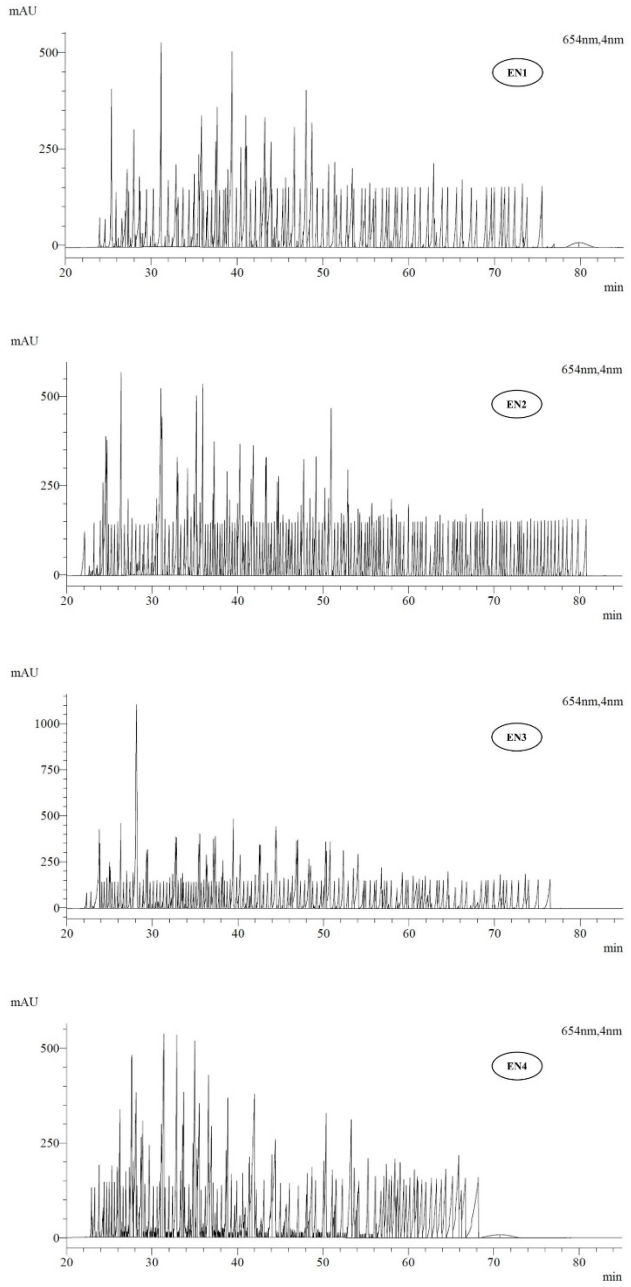

Figure 3: HPLC spectra of all four different aqueous extracts of *E. foetidum* leaves at 654 nm, 4 nm.

Phytochemicals Qualitative Analysis

All four aqueous extracts of *E. foetidum* leaves from four different extraction techniques contained essential phytochemicals such as saponins, terpenoids, alkaloids, polyphenolics, tannins, glycosides, flavonoids, phytosterols, coumarins, quinones, and betacyanins, but not chalcones and anthocyanins. The availability of phytochemicals in all four different aqueous extracts of *E. foetidum* leaves was also supported statistically by statistical analysis using the non-parametric test. Statistical analysis revealed no significant variation in the presence of essential phytoconstituents examined in the four distinct aqueous extracts recovered from *E. foetidum* leaves using four different extraction methods at the 5% significant level. Since no variation in the qualitative analysis of the phytoconstituents was found at the 5% significant level, this study revealed that any of these extraction techniques may be employed to extract phytoconstituents.

Phytochemical Quantitative Analysis

The quantification of AC, TPC, TC, TTC, SC, and TFC in all four distinct aqueous extracts recovered from *E. foetidum* leaves by four different extraction methods exhibited varied quantities, as shown in Table 4. The findings demonstrated the presence of a variety of phytoconstituents in *E. foetidum* leaves, with the SC, TPC, TC, and TTC accounts showing to be greater in the extraction method EN4 (185.84 ± 0.54 mg SE/g, 37.37 ± 0.65 mg GAE/g, 0.89 ± 0.01 mM LE/g, and 36.99 ± 0.64 mg TAE/g, respectively) and smaller in the method EN1. TFC values in extraction technique EN2 were high (11.84 ± 0.14 mg QE/g), but low in extraction technique EN3. In contrast, AC was revealed to be greater in extraction approach EN3 (1.67 ± 0.01 mg AE/g) and lower in extraction approach EN2. As shown in Figure 4, the statistical analysis, the T-test (LSD), strongly disclosed that all of the extraction approaches employed in this research extracted significantly various amounts of saponins, polyphenolics, alkaloids, terpenoids, flavonoids, and tannins at the 5% significant level.

This study demonstrated that when extracting polyphenolics, tannins, saponins, and terpenoids from the leaves of *E. foetidum* using water as a solvent, the EN4 extraction technique is the best method. The EN4 extraction process can extract the majority of significant phytochemicals. The extraction technique EN2 can extract a greater quantity of flavonoids than the other extraction methods utilized in this study. The soxhlet extraction method (EN2) has previously been shown to be a highly effective way to extract flavonoids from medicinal herbs (Kokilananthan et al., 2022a; Kokilananthan et al., 2022b). The current research also found that the extraction technique EN3 is an excellent approach for extracting alkaloids, as previously reported (Kokilananthan et al., 2022a; Kokilananthan et al., 2022b).

Table 4: Phytochemical quantitative analysis data of aqueous extracts from four different extraction approaches utilized for extracting phytochemicals from *E. foetidum*. The values represent the mean and standard deviation of triplicate samples.

Phytochemicals	Extraction Techniques			
	EN1	EN2	EN3	EN4
Polyphenolics content (mg GAE/g)	19.73 ± 0.06	30.00 ± 0.40	24.87 ± 0.50	37.37 ± 0.65
Flavonoids content (mg QE/g)	6.33 ± 0.04	11.84 ± 0.14	5.97 ± 0.03	9.22 ± 0.17
Tannins content (mg TAE/g)	19.53 ± 0.05	29.69 ± 0.40	24.61 ± 0.50	36.99 ± 0.64
Terpenoids content (mM LE/g)	0.51 ± 0.00	0.79 ± 0.02	0.67 ± 0.01	0.89 ± 0.01

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Saponins content (mg SE/g)	143.22 ± 0.41	171.08 ± 0.90	150.13 ± 0.74	185.84 ± 0.54
Alkaloids content (mg AE/g)	1.61 ± 0.01	0.66 ± 0.01	1.67 ± 0.01	0.70 ± 0.01

Samples were collected at Bukit Ulu Piah, Tambun, Perak. Bukit Ulu Piah is a lowland, secondary dipterocarp forest in the Kinta district, Perak, situated at 4° 35' 59" N, 101° 09' 59" E. The highest peak of Bukit Ulu Piah stands at an altitude of 175m above sea level. The sampling site comprised vegetated areas, including grasslands, fenced plants, understory vegetation in palm oil tree areas, and slopes covered with shrubs, saplings, tall trees and grass on both sides of the vehicle path to the hill summit.

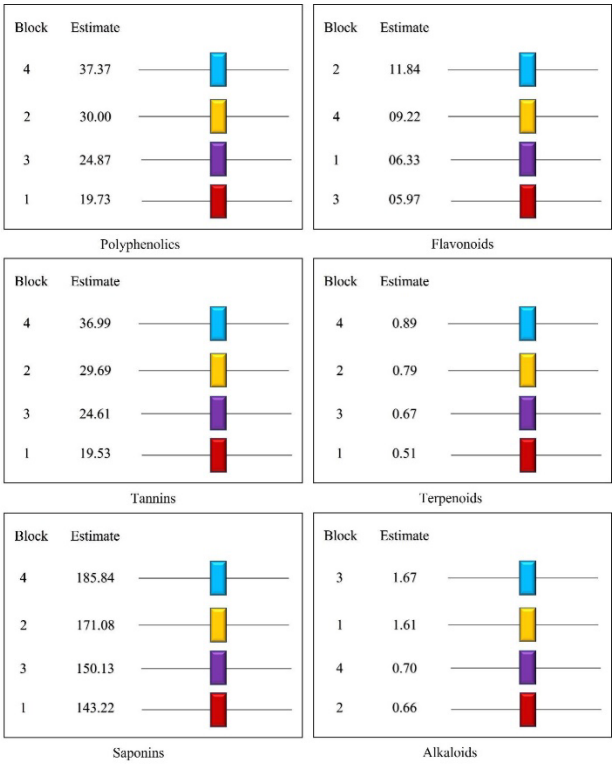

Figure 4: Data from statistical analysis of phytoconstituents quantification of four main extraction methods of *E. foetidum* leaves' aqueous extracts. (Block: 1: EN01, 2: EN02, 3: EN03, 4: EN04, Alpha = 0.05, Estimate: The mean values covered by the same bar do not differ significantly).

Antioxidant Analysis

The antioxidant capacity was seen in all four aqueous extracts of *E. foetidum* leaves prepared using the four different extraction techniques, but it varied greatly between techniques. The total antioxidant power determined by the FRAP assay revealed that the extraction approach EN4 (47.17 ± 0.20 mg Trolox Eq/g) has a greater capacity for antioxidants than all other aqueous extracts of *E. foetidum* leaves. Most notably, all employed extraction approaches have shown significant differences at the 5% significant level, as illustrated in Figures 5 and 6.

Figures 5 and 6 illustrate the results of DPPH radical scavenging effect, which revealed that extraction procedure EN3 (12.91 ±0.02 mg/ml) has relatively higher levels of radical scavenging capacity than the other three methods. However, the radical scavenging capacity of four distinct *E. foetidum* leaf aqueous extracts is substantially lower than that of the standard used, ascorbic acid (139.05 ± 0.05 ppm). Additionally, statistical results revealed that all four aqueous extracts from *E. foetidum* leaves did not have the same scavenging capability at the 5% significant level. The results showed that all four extraction methods utilized in this study resulted in various degrees of antioxidant potential with *E. foetidum* leaves. As a result, the extraction approach EN4 is a well-suited method in all aspects of analysis. According to the current study, extraction methodology EN4 is a well-suited method in every aspect.

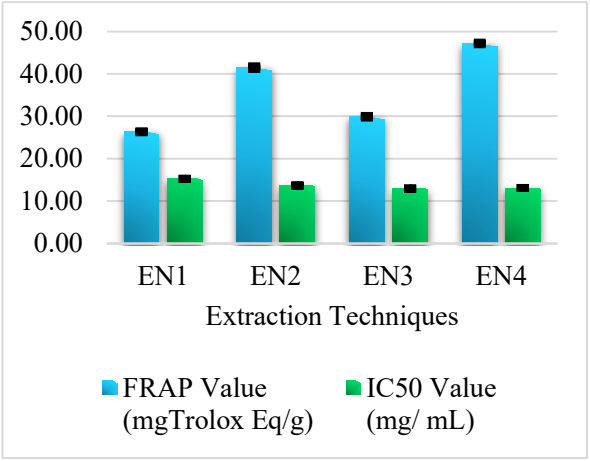

Figure 5: Comparison of antioxidant potential of four different aqueous extracts of *E. foetidum* leaves using FRAP and DPPH assays.

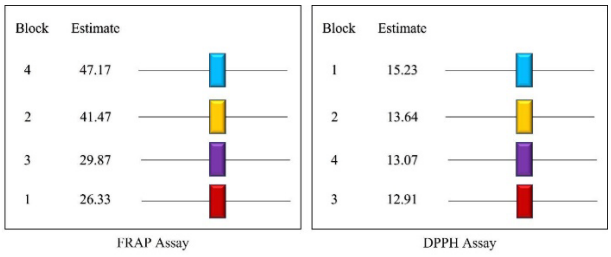

Figure 6: Data from statistical analysis of the antioxidant potential of four distinct extraction methods of *E. foetidum* leaves' aqueous extracts. (Block: 1: EN1, 2: EN2, 3: EN3, 4: EN4, Alpha = 0.05, Estimate: The mean values covered by the same bar do not differ significantly).

As a result, the HPLC application is also detailed, as other phytochemical quantitative and antioxidant analysis results show that EN2 and EN4 are the best extraction methods to extract most of the phytochemicals from the leaves of *E. foetidum*. Notably, while the number of chemical constituents was higher in the extraction technique EN2 than in the extraction technique EN4 based on the HPLC spectra, the antioxidative potential by FRAP assay was higher in the extraction technique EN4. Likely, other

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quantified phytochemicals, except for flavonoids, were found to be higher in the EN4 extraction technique, whereas the second highest was observed in the EN2 extraction technique, but interestingly, flavonoids were found to be the most abundant in EN2. As a result, this study strongly suggests that the extraction techniques EN2 and EN4 are the best methods for extracting phytochemicals, with EN4 being the most recommended when EN2 and EN4 are compared. Notably, this is the first comprehensive study comparing aqueous extraction techniques with spectrophotometric and HPLC analyses.

4. Conclusion

This study concludes that the EN4 extraction method (maceration with agitation at 60ºC) effectively extracts antioxidants, saponins, polyphenolics, terpenoids, and tannins from *E. foetidum* leaves. The EN2 method (Soxhlet at 105ºC) is optimal for flavonoids, while EN3 (maceration with agitation at room temperature) excels in alkaloid extraction. Although *E. foetidum* leaves have a broad range of bioactive constituents and higher antioxidant properties, the quantity of phytoconstituents and antioxidant potential varies on extraction method. HPLC is the preferred method for analyzing crude phytochemical profile of crude extracts. Thus, choosing the right extraction process is crucial in natural product isolation.

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