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Original Article

MICROWAVE ASSISTED EXTRACTION; PHYTOCHEMICAL EVALUATION OF MALAYSIAN PALM OIL TRUNK EPIPHYTES FERNS

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ABSTRACT

Objective: The objective of this research was to evaluate the, total phenolic contents (TPC), total flavonoid contents (TFC), antioxidant activity, cytotoxicity and functional group of epiphytes ferns grows on the Malaysian palm oil trunk (MPOTEF): that includes *Nephrolepis biserrata* (NBF), *Davallia denticulata* (DDF), *Asplenium longissimum* (ALF), *Gonioplebium percussum* (GPF), *Stenochlaena palustris* (SPF), *Vittaria elongata* (VLF) and *Vittaria ensiformis* (VSF).

Methods: For extraction, microwave oven assisted method was used. TPC was determined by following the Folin-Ciocalteu colorimetric method and TFC was determined using aluminium chloride colorimetric assay. Antioxidant activity was determined by DPPH-scavenging assay methods.

Results: All the ferns exhibited good results of TPC, TFC and antioxidant activity. SPF showed highest TPC and TFC in aqueous extracts; 639.4 mg/g and 172.71 mg/g respectively, and the same result showed in ethanol extracts; 271.61 mg/g and 174.54 mg/g, respectively. SPF also giving scavenged the free radicals 94.85% in aqueous extract while 98.17% in ethanol extract. The brine shrimp cytotoxicity revealed DDF having the strongest result (130µg/ml (dry mass) in ethanol extract as compared to in water extract and other species. The FTIR indicated the presence of alcohols, phenols, amine, alkanes, alkenes, alkyl halides, carbonyl, nitro compounds, acid, ether and ester in different species and extracts. Prediction Activity Spectra of Substances (PASS) program for SPF extract showed the most probable activities are antioxidant, lipid peroxidase inhibitor and radical scavengers.

Conclusion: All the ferns showed active toxicity in ethanol extract whereas inactive in the water extracts except SPF. Amongst them, SPF shows its capability as a natural antioxidant source appears to be an alternative to synthetic antioxidants. It can be seen from the results of PASS for SPF extract that most probable activities are antioxidant, lipid peroxidase inhibitor and radical scavengers supported by cytotoxicity and FTIR results. Thus, the present approach can be very useful in fern prediction activity according to their required properties.

Keywords: Epiphytes fern, Palm oil, Total phenolic compound, Total flavonoid compound, Antioxidant activity, Brine shrimp lethality test, FTIR

INTRODUCTION

Several fern species have an established history for being used for various ailments in traditional medicine. Extraction of phytochemicals ingredients from ferns plays an effective role in the drug development process [1, 2]. A myriad of studies indicates that a special category of ferns, known as medicinal ferns, contain a variety of naturally existing antioxidants, such as phenols and flavonoids, which are well-known in their antitumor, antimutagenic and antibacterial properties [3].

The extraction process is an important step for separation and evaluation of active components in various ferns [4, 5]. Generally, the extraction procedure is chronological and analytically conceded out using aprotic and nonaprotic solvents to extract polyphenolic compounds in samples [2]. Thus, with the intention of recovery of the significant yield of phytoconstituents, several researchers were used different extraction techniques such as solid-liquid, liquid-liquid include traditional solvent extraction, Soxhlet extraction, and supercritical fluid extraction, ultrasonic extraction and microwave-assisted extraction (MAE) [6]. Among all extraction technique, MAE is a relatively outstanding method used for the extraction of natural products [7, 8].

The brine shrimp lethality test is a simple bioassay for a natural product. The research was considered as a useful tool for preliminary assessment of cytotoxicity and for pharmacological activities screening in fern extracts [9]. Brine shrimp larvae have been used as a bioassay for a variety of toxic substances. The technique is easily mastered, costs little, and utilizes a small amount of test material [10]. Nevertheless, an evaluation of polyphenolic compound for their antioxidant capacity and cytotoxicity among several species of epiphytes fern in Malaysian palm oil plantation is still needed to explore.

The FTIR method was performed on a spectrophotometer, which can be used to detect peak values and their functional groups. FTIR is one of the most widely used methods to identify the chemical constituents and elucidate the compound structures in order to propose in medicinal purposes [11, 12]. Previous researchers carried out the FTIR in order to notice the minor changes of primary and secondary metabolites [13, 14], to recognize the concrete structure of certain plant secondary metabolites [15] and characterize functional groups that are responsible for various medicinal properties of herbal plants [16, 17].

Therefore, in this study, we are keen to help the pharmaceutical industry to explore new natural antioxidant compounds through the implementation of temperature controlled microwave assisted extraction (TCMAE) in water and ethanol of active medicinal ingredients from different Malaysian palm oil trunk epiphytes fern (MPOTEF) materials.

MATERIALS AND METHODS

Reagents and solvents

Sodium hydroxide, α-tocopherol, butylated hydroxyl anisole (BHA) were purchased from Sigma-Aldrich (Missouri, US). Gallic acid, sodium carbonate, aluminium chloride 1,1-diphenyl-2-picrylhydrazyl (DPPH), Folin-ciocelteau, sodium nitrite, catechin, acetonitrile, acetone, and ethanol were purchased from Merck (New Jersey, US). All the reagents were of analytical grade.

Apparatus and instruments

Temperature controlled microwave extraction system (TCMES), thermocouple Type K, Thermocouple data logger (TC-08, Pico Technology), rotary vacuum evaporator (Buchi, Germany), freeze drier (Cleanvac 8 BIOTRON, South Korea), cuvette, oven (Memmert, Germany), sonicator (Branson, USA), UV-Vis spectrophotometer (Thermo Scientific model Genesys 10S).

Fern materials

The seven species of epiphytes ferns were collected from palm oil trunk at palm oil plantation at Seri Medan, Johor Malaysia, in August 2012. The species were identified by Mr. Muhd Ruzi, Research Officer in Faculty of Science and Technology, National University of Malaysia (UKM), the voucher specimens labeled in table 1 have been deposited in the Herbarium of the Rimba Ilmu, the University of Malaya for further reference. General information and voucher details of the used ferns can be seen in table 1.

Preparation of TCMAE of epiphytes ferns

In the microwave-assisted extractions method, the highly dielectric properties solvent (water and ethanol) were used. In this study, the extraction system was developed by modification of the domestic microwave oven (Haier, model EA-180M, Serial number: 6921140329155). It had a rated power output of 700 watts (5.4A, 230-240 V, 50 Hz) with an operation frequency of 2450 MHz. The microwave oven was modified by making 2 holes with a diameter less than 7 cm to ensure safety and also to accommodate the two-necked vessels with the fluid sealed stirring device [21]. Each species of epiphytes fern was initially dried in the microwave oven for about 10 min at a medium high pulse (420 watts/50 °C) to remove moisture from 10-11% moisture content.

The dried fronds of epiphyte ferns were ground by Panasonic MX896TM grinder. They were passed through a stainless steel, sieve with a pore size of 0.3 mm (Impact Laboratory Test Sieve 850 MIC aperture) before extraction. Subsequently, the fern material (16.5 g) was accurately weighed into a reaction vessel, to which was added 500 ml of water placed in the glass vessel set up equipped with glass connectors attached to the reflux condenser and a thermocouple to control the temperature. In order to increase the possibility of interaction between the materials inside the reaction vessel and microwave's radiation and to maximize absorption, the fern material and solvent water are placed in a reaction vessel which is continuously stirred to ensure uniform extraction. The sample was extracted using temperature controlled microwave extraction system (TCMES) at 70 °C in 4 min of reaction and 560 Watts microwave power. Then the solution was filtered using Whatman 41 to get supernatant. The supernatant dried by freeze dryer. The sample was stored in a-20 °C for further use.

Table 1: Collection and identification of Malaysian palm oil trunk epiphytes ferns (MPOTEF)

Order	Family	Genus	Species	Common name		Specimen No.	Voucher No.
				English	Malay		
Polypodiales	Polypodiaceae	*Nephrolepis*	*biserrata*	Giant sword fern	Paku uban, paku larat	FK001	KLU47725
Filicales	Davalliaceae	*Davallia*	*denticulata*	Rabbit's foot fern	Paku tertutup, Sakat laipang	FK002	KLU47726
Polypodiales	Polypodiaceae	*Goniophlebium*	*percussum*	Little foot	Paku lempai	FK003	KLU47727
Blechnales	Blechnaceae	*Stenochlaena*	*palustris*	Miding fern	Paku miding, paku udang	FK004	KLU47728
Polypodiales	Aspleniaceae	*Asplenium*	*longissimum*	Spleenwort	-	FK005	KLU47729
Pteridales	Vittariaceae	*Vittaria*	*Elongate*	Shoestring fern	-	FK006	KLU47730
Pteridales	Vittariaceae	*Vittaria*	*Ensiformis*	Tape fern	-	FK007	KLU47731

Antioxidant properties analysis

Determination of TPC

TPC of TCMAE water extracts was carried out using Folin–Ciocalteu reagent according to the reported method [18]. The concentration of polyphenol was calculated from the calibration curve using gallic acid, and the results were expressed in gallic acid equivalents (GAE mg/100 gm dry weight material). Samples were analyzed in triplicates.

$$Total\ \ phenolic\ compound\ (TP) = \frac{C \times V \times df}{M} \quad \ldots. (1)$$

Where:

C = gallic acid (µg/ml) (i. e 0-200 µg/ml)

V = volume of plant extract (ml)

M = sample weight (mg)

df = dilution factor

Determination of TFC

TFC of TCMAE water extracts was determined by the aluminium chloride colorimetric method by [9, 20]. The flavonoid was calculated based on the catechin calibration curve, and the result was expressed in catechin equivalents (mg CE/g dry weight material).

Measurement of DPPH radical scavenging activity (% inhibition) and IC_{50}

The radical scavenging activity of epiphytes species was estimated according to the procedure modified by [22]. IC_{50} value was determined from the plotted graph of scavenging activity against the concentrations of TCMES water extracts of each species epiphytes fern samples, which is defined as the concentration of extract causing 50% inhibition of absorbance. Triplicate measurements in different concentration were carried, such that a 50% fall in absorbance of the DPPH can be calculated. The IC_{50} value of each extract was measured and compared with the corresponding α-tocopherol and butylated hydroxyl anisol (BHA).

Cytotoxicity assay

Brine shrimp lethality bioassay was carried out to investigate the cytotoxicity of water and ethanolic extracts of all ferns [23]. Brine shrimps were hatched using brine shrimp eggs in a conical shaped vessel, filled with sterile artificial seawater (prepared using sea salt 38 g/l and adjusted to pH 8.5 using 1N NaOH) under constant aeration for 48 h. After hatching, active nauplii free from egg shells were collected from a brighter portion of the hatching chamber and used for the assay. Ten nauplii were drawn through a glass capillary and placed in each vial containing 4.5 ml of brine solution. In each experiment, 0.5 ml of the fern extract was added to 4.5 ml of brine solution and maintained at room temperature for 24 h under the light and surviving larvae were counted. Experiments were conducted along with control (vehicle treated), different concentrations (1-5000 µg/ml) of the test substances in a set of three tubes per dose. The percentage of mortality (% M) was calculated as % M = percentage of survival for the control percentage of survival in the treatment. From this data, the percent of lethality of the brine shrimp nauplii for each concentration and control was calculated. An approximate linear correlation was observed when the logarithm of concentration versus percentage of mortality was plotted on the graph paper, and the values of IC_{50} were calculated using Microsoft Excel 2003.

Fourier transforms infrared spectrophotometer (FTIR)

Dried powder of different solvent extracts of each plant materials was used for FTIR analysis. A dried extract powder was encapsulated in KBr pellet, in order to prepare translucent sample

discs. The powdered sample of each plant specimen was loaded in FTIR spectroscope (Bruker IFS 66/S), with a scan range from 450 to 4000 cm^{-1}.

Statistical and data analysis

The mean values of the data from each species were compared using one-way analysis of variance (ANOVA) using SPSS (version 18) for Windows (SPSS Inc, Chicago, IL, USA) with P-value of less than 0.05 was considered significant. Regression linear was used to correlate the relationships between antioxidant activities, TPC and TFC were calculated using Microsoft Excel 2003.

RESULTS AND DISCUSSION

The total phenolic content (TPC) of the seven species of MPOTEF in water and ethanol extracts are summarized in table 2. SPF (639.4 mg GAE/g dry weight) contained the highest average of TPC in water extract followed by VLF>VSF>DDF>GPF>NBF and>ALF. On the other hand, SPF (271.60 mg GAE/g dry weight) also contained the highest average of TPC followed by VLF>VSF>GPF>NBF>ALF and>DDF in ethanol extract.

Study on the effect of fertilizer on TPC was done by the previous researcher [24-26]. Hence, the higher amount of TPC in SPF, VLF and VSF in this study might be attributed to their fertilizer. Among the other epiphytes fern, SPF, VLF and VSF are growing at the bottom of palm oil, which contacted indirectly to the fertilizer that put on the base of palm oil fern. Moreover, the result as shown in the Supplementary result (S-1; Supplementary information) were also in agreement with [27] which revealed the TPC was considerably decreased by increasing nitrogen fertilization whereas increased by increasing sulfur supply. It provides clear evidence that nitrogen and sulfur nutrition was manipulated the total phenolic concentrations of epiphytes fern in such a way improved the nutritional value of these epiphytes fern.

In addition, TPC value in water extract is higher as contrasted to ethanol extract. The presence of carbonyls and organic acids can dissolve in water rapidly as compared to ethanol at the presence of microwave irradiation due to their favorable polarity and uniform solvent temperature. The extraction was done in temperature controlled microwave closed system and supported by fluid sealed stirring was provided a consistent temperature during the extraction process.

Table 2: TPC, TFC and DPPH assay (% inhibitory and IC_{50}) of MPOTEF by TCMES

Epiphytes	Water Extracts				Ethanol Extracts			
Ferns	TPC	TFC	Inhibition	IC_{50}[a]	TPC	TFC	Inhibition	IC_{50}[a]
	(mg GAE/g)	(mg CE/g)	%	(µg/ml)	(mg GAE/g)	(mg CE/g)	%	(µg/ml)
NBF	199.4+2.0	52.71+2.4	86.67+0.15	215.67+11.2	119.6+0.7	152.77+2.5	48.12+0.27	723.27+12.0
DDF	417.4+5.3	112.5+2.0	92.73+0.25	65.14+9.8	45.6+2.3	137.17+2.0	33.57+0.15	897.76+15.2
ALF	175.4+1.2	20.71+2.8	86.36+0.15	235.98+7.6	95.6+0.6	170.77+3.1	55.79+0.34	775.63+10.0
SPF	639.4+3.1	172.71+2.7	94.85+0.10	40.03+4.7	271.6+0.8	274.77+0.8	98.67+0.27	145.43+8.7
GPF	289.4+4.2	61.57+1.3	91.01+0.06	165.55+11.5	97.6+0.4	115.97+1.2	62.6+0.12	861.56+10.4
VLF	445.4+1.2	115.57+1.0	92.83+0.25	82.72+4.4	125.6+1.9	132.37+2.2	73.01+0.30	528.93+12.2
VSF	431.4+2.0	117.57+1.3	93.74+0.25	107.97+13.4	117.6+2.3	178.37+3.2	56.49+0.32	512.89+14.5
Standard								
α-tocopherol	-	-	98.39±0.25	47.79+8.2	-	-	98.39±0.25	47.79+8.2
BHA	-	-	98.72±0.8	9.25+3.7	-	-	98.7±0.8	9.25+3.7

Davallia denticulata (Burm.) Mett. (DDF), *Nephrolepis biserrata* (Sw.) Schott. (NBF), *Asplenium longissimum* B1(ALF), *Gonioplebium percussum* (Cav.) *Wagner & Grether* (GPF), *Stenochlaena palustris* (Burm. f.) Bedd (SPF), *Vittaria Elongata* (VLF), *Vittaria ensiformis* (VSF).

[a]Values obtained from regression lines with 95% of confidence level. IC_{50} is defined as the concentration sufficient to obtain 50% of a maximum inhibition.

The total flavonoid content (TFC) values of the water and ethanol extract of each MPOTEF are summarized in table 1. TFC of TCMA water extract epiphytes fern varied range from 20.71 to 172.71 mg CE/g of dry weight material. TCMA water extract of SPF (172.71 mg CE/g dry weight) contained the highest average of TFC followed by DDF (88.37 mg CE/g dry weight), VSF (117.57 mg CE/g dry weight), VLF (115.57 mg CE/g dry weight), GPF (61.57 mg CE/g dry weight), NBF (52.71 mg CE/g dry weight), and the lowest flavonoid content was found of ALF (20.71 mg CE/g dry weight).

As shown in table 1, ethanol extracts revealed a higher recovery as compared to water extracts. VSF (178.34 mg CE/g dry weight) contained the highest average of TFC in ethanol extract followed by SPF (174.77 mg CE/g dry weight), ALF (170.77 mg CE/g dry weight), NBF (152.77 mg CE/g dry weight), DDF (137.17 mg CE/g dry weight), VLF (132.37 mg CE/g dry weight), and GPF (115.97 mg CE/g dry weight), respectively. It is suggested that the ethanolic solvent is having the tendency to be dissolved a different aliphatic, aromatic and polycyclic aromatic compounds inside the microwave closed system. Flavonoids compounds usually consist of aglycone or glycosylated forms, located in the vacuoles within fern cells and are in the polar soluble fraction. Therefore, flavonoids can be easily extracted under temperature controlled microwave assisted (TCMA) with ethanol, which is insoluble lignins and tannins that bind to proteins on plant cell were disrupted during the extraction.

The results of the scavenging effect of TCMES in both extracts for seven species of epiphytes fern on DPPH radical are also given in table 2. The effect of antioxidants on DPPH is thought to be due to their radical scavenging and hydrogen donating ability which could provide as free radical inhibitors or scavengers. Through this assay, it was observed that the violet colour of DPPH was reduced to a pale yellow colour due to the abstraction of the hydrogen atoms from antioxidant compound [28]. The more antioxidants occurred in the extract; the more DPPH reduction will occur. It was observed that the highest % inhibition for DPPH radical scavenging activity of water extract is SPF (94.85%) while the lowest is ALF (86.36%). In ethanol extract, SPF also showed highest inhibition values which is 98.65% among all MPOTEF species, and it was followed by VLF (73.01%), GPF (62.6%), VSF (56.49%), ALF (55.79%), DDF (33.57%), and NBF (48.12%). The result of SPF in ethanol extract was also higher than standard antioxidants such as α-tocopherol (98.39%) and BHA (98.72%).

High reduction of DPPH is related to the high scavenging activity performed by the particular sample as reported by [29]. Since IC_{50} is a measure of inhibitory concentration, a lower IC_{50} value would reflect greater antioxidant activity for the sample. As shown in table 1, IC_{50} of water extract in a range from 40.03 µg/ml to 235.98 µg/ml. SPF (40.03 µg/ml) contained the lowest number followed by DDF (65.14 µg/ml), VLF (82.72 µg/ml), VSF (107.97 µg/ml), GPF (165.55 µg/ml), NBF (215.67 µg/ml), ALF (235.98 µg/ml).

On the other hand, the IC_{50} of each MPOTEF species in ethanol was in the range 145.43 µg/ml to 897.76 µg/ml where was found to be lower antioxidant activity as compared to in water extract. The IC_{50} showed SPF contained the higher antioxidant activity followed by VSF>VLF>NBF>ALF>GPF>DDF. In addition, IC_{50} values of the

synthetic antioxidant BHA was found the more active with an IC_{50} value of 13.15 µg/ml while, for the natural antioxidant α-tocopherol also shows more active (60.05 µg/ml) as compared to all epiphytes fern. The trend is same as shown by [30].

The study on % DPPH radical scavenging activity and IC_{50} of MPOTEF revealed that ethanol extract results were lower than water extract. Water indeed, possesses a much higher dielectric constant than ethanol (80 versus 24.6). For this reason, water proved more suitable for use with microwave irradiation, resulting in a more rapid temperature increase and better extraction efficiency. It could be that some of the target compounds of these ferns were purified and isolated in water extract which had inhibitory roles in the test experiments might be not dissolved properly in ethanol extract.

Correlation of DPPH scavenging activity with TPC and TFC

The correlation between antioxidant capacity with TPC and TFC of seven species MPOTEF in water and the ethanolic extract was illustrated in fig. 1 and fig. 2 respectively. The experimental results observed that TPC and TFC of MPOTEF had strong correlation effect on antioxidant activities of crude extract in both extracts. These results were also in agreement with [31] which showed a close relationship between TPC and TFC with the antioxidant activity of black currant leaf while another researcher also reported the same correlation analysis on different plants [32, 33, 34]. These results were also consistent with the finding of [35] who reported a good correlation of TPC in red fruit but dissimilar the results of [36] and [37] who did such a weak correlation.

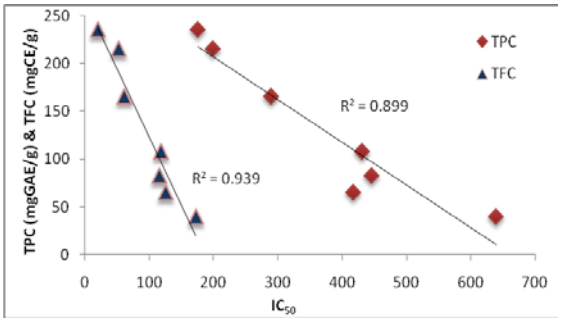

Fig. 1: Relationship between total phenolic content (mg GAE/g) and total flavonoid content (mg CE/g) with antioxidant capacity (IC_{50}) of water extract MPOTEF

A good correlation between TPC and TFC with DPPH scavenging activity indicated that the extracts obtained from epiphytes fern have remarkable antioxidant activities. It is could be suggested that the composition of phenolic and flavonoids compounds is a key determinant of the radical scavenging activity. This is also implying that the antioxidant activity of water and ethanolic extract of MPOTEF depends on the numbers and positions of the hydroxyl groups in relation with the glycosyl group and due to the presence of polyphenolics, carbonyl compounds. However, it also can be stated that scavenging effect of extracts is not limited to phenolic and flavonoid compounds. Moreover, the antioxidant activity of a MPOTEF does not rely exclusively on phenolic compounds, but also on other substances such as volatile oil, carotenoids, vitamins, and minerals. Synergistic effects may also take place between different types of antioxidants. These differences in antioxidant activities of fern extracts may be due to miscellaneous qualitative and quantitative compositions of phenolic and flavonoids components [38].

As shown in fig. 1 and 2, the correlation between antioxidant capacity with TPC of both extract: water (R_2= 0.899) and ethanol extract (R_2=0.723) was lower than the TFC of water (R_2=0.723) and ethanol extract (R_2=0.862) respectively. These results suggested that ethanol extract of flavonoid compounds contributed a higher antioxidant capacity (IC_{50}) of the extract as compared to the phenolic compound of MPOTEF.

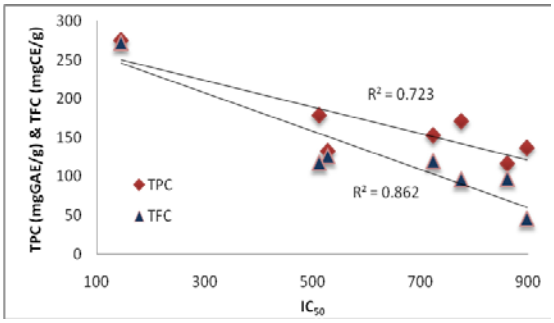

Fig. 2: Relationship between total phenolics content (mg GAE/g) and total flavonoid content (mg CE/g) with antioxidant capacity (IC_{50}) of ethanol extract MPOTEF

Correlation of TPC with TFC

Analysis of TPC and TFC of MPOTEF was shown in fig. 3 revealed a good correlation in water (R^2=0.958) and ethanolic (R^2=0.768) extracts respectively, at a fixed temperature, as already shown by other authors for pe gaga [39]. Since flavonoids are a group of fern phenolics, this finding suggested that the phenolic compounds found in epiphytes fern may be mainly flavonoids.

It is generally considered that extraction at a prominent temperature preferably 70 °C will lead to a significant amount of extraction yield of bioactive compounds especially in a microwave closed system [40]. Our records also implied that TCMAE of MPOTEF at a fixed temperature in two solvents has different effects on the yields of bioactive components, although they may share some uniform constituents such as the amount of antioxidants. Particularly, TCMES increased extraction of TPC and antioxidant activity in water by enhancing both diffusion coefficient and solubility of the extract [41]. Moreover, higher dielectric properties of water will increase cell membrane permeability through the breakdown of cellular constituents of fern cells which lead to releasing of bound polyphenols in plants during extraction. [42-44].

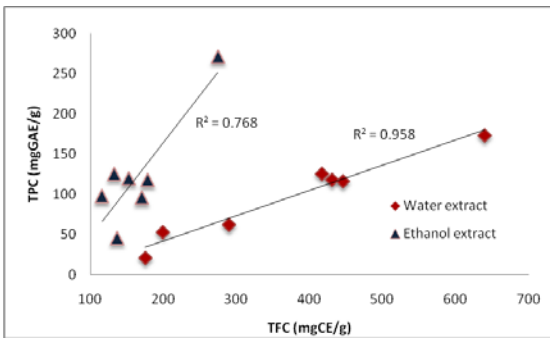

Fig. 3: Relationship between total phenolics (mg GAE/g) with total flavonoid (mg CE/g) of water and ethanol extract of MPOTEF

PASS study

Among all the seven species, the SPF shows the outstanding result in phenolic, flavonoid and antioxidant activity in both solvent. In this regard, an effort has been made to explore and confirm more pharmacological profile for the phyto constituents of SPF extract by using computer-aided drug discovery program PASS. PASS prediction tools are constructed using 20,000 principal compounds from the MDDR database (produced by Accelrys and Prous Science) [45]. In our study, the antioxidant, lipid peroxidase inhibitors, and radical scavenging properties on the basis of the structural formula of SPF were obtained by using PASS as shown in (table 3). The result of prediction is presented as the list of activities with appropriate Pa

and Pi ratio. Pa and Pi are the estimates of probability for the compound to be active and inactive, respectively. It is reasonable that only those types of activities may be revealed by the compound, which Pa>Pi. If Pa>0.3 the compound is likely to reveal this activity in experiments, but in this case, the chance of being the analog of the known pharmaceutical agents for this compound is also high. Thus, potential biological effects of the fern constituents were predicted by PASS program based on structure-activity relationship (SAR) analysis of the training set containing thousands of compounds which have many kinds of biological activity. Therefore, to confirm and strengthen our results in the experiments, only we used PASS program to approve the antioxidant, radical scavenging and lipid peroxidase inhibitor activities of SPF constituents as shown in table 3. The results are strongly in agreement with our experiment.

Table 3: Part of the predicted biological activity spectra for the chemical compounds of SPF extract Pa—probability "to be active"; Pi—probability "to be inactive"

Compound	Antioxidant		Radical scavenging activity		Lipid peroxidase inhibitor	
	Pa	Pi	Pa	Pi	Pa	Pi
1	0.822	0.003	0.987	0.001	0.942	0.002
2	0.804	0.003	0.995	0.000	0.973	0.002
3	0.805	0.003	0.984	0.001	0.977	0.002
4	0.809	0.003	0.983	0.001	0.933	0.002
5	0.801	0.003	0.946	0.001	0.940	0.002
6	0.678	0.004	0.981	0.001	0.887	0.003
7	0.433	0.010	0.301	0.030	0.450	0.024
8	0.387	0. 013	0.373	0.020	0.433	0.027

Brine shrimp (Artemia salina) lethality assay

The LC_{50} brine shrimp (artemia salina) lethality test of seven species of MPOTEF evaluated in this screening are summarized in table 4. All of the epiphytes fern ethanol extract tested showed the medium result of brine shrimp larvicidal activity according to [23] who classified crude extracts and pure substances into toxic (LC_{50} value<1000 µg/ml) but not for water extract, which showed poor or non-toxic activity because all the extract having a value of LC_{50} more than 1000 µg/ml except for SPF. It is believed that fern extracts with low LC_{50} values possibly will have metabolites with cytotoxic, antifungal, insecticidal or pesticide activities, which contribute to the toxicity of the ferns. The LC_{50} values of ethanol extract for NBF, DDF, ALF, GPF, SPF, VLF, VSF were 340 µg/ml, 130 µg/ml, 220 µg/ml, 430 µg/ml, 440 µg/ml, 400 µg/ml and 430 µg/ml, respectively.

Table 4: Brine shrimp cytotoxicity of water and ethanol extract of MPOTE

Epiphytes	IC_{50} Water	IC_{50} Ethanol
Ferns	µg/ml	µg/ml
NBF	>1000	340
DDF	>1000	130
ALF	>1000	220
SPF	825	430
GPF	>1000	440
VLF	>1000	400
VSF	>1000	430

However, this amount as compared to other reported, medicinal fern was considered medium value. The medium or poor LC_{50} value of both extracts might be due to factors like temperature variations during the process which force denatured some of the active compounds in the fern and another reason these ferns may be active only when it's fresh.

Although some of these epiphytes ferns had similar secondary metabolites, their toxicity against the brine shrimp larvae was seen to be varying. According to [46] the brine shrimp test is not enough to disqualify the medicinal values of these fern's variation. The quantitative analysis only gives a rough guide about what the extracts contain. He elaborated that some of the compounds are in such tiny amounts that cannot be detected by this method. This statement gave a good explanation behind our results of toxicity in water extract. In this research, water extract showed LC_{50} value>1000 µg/ml for all species except SPF 825 µg/ml.

The cytotoxic property by SPF in both solvents might be due to the presence of a good amount of flavonoid content. A significant amount of flavonoids in fern material has been reported to possess many useful properties, including anti-inflammatory activity, oestrogenic activity, enzyme inhibition, antimicrobial activity [47, 48], antiallergic activity, antioxidant activity [49], vascular activity and cytotoxic antitumor activity [50] which reflected cytotoxicity study.

In the current study, we found that all the seven species of MPOTEF ethanol extract used for this study showed significant toxicity against brine shrimp larvae of Artemia salina at lower LC_{50} values. The results obtained from the brine shrimp lethality bioassay of epiphytes fern can be an indicative of the presence of potent cytotoxic components, which warrant further investigation.

Fourier transforms infrared spectrophotometer (FTIR)

The data on the peak values and the probable functional groups of seven species of MPOTEF in water and ethanol extract attained by FTIR analysis presented in table 5. The FTIR spectrum was tabulated in Supplementary S2 for further reference. Results of FTIR spectroscopic studies have revealed the presence of a various chemical constituent in the aqueous and ethanolic extract of seven species of MPOTEF. The FTIR spectrum confirms the presence of alcohols, phenols, amine, alkanes, alkenes, alkyl halides, carbonyl, nitro compounds, acid, ether and ester in different species and extracts. These functional groups are responsible for various medical properties [51, 52].

Among the functional groups observed in the extracts, OH group was found to be present in all MPOTEF in both solvents. As OH group has got the ability of forming hydrogen bonding capacity, presence of OH group probably indicates the higher potential of water and ethanol extract towards inhibitory activity against microorganisms [12]. This agrees with the result of antimicrobial activity analysis as shown in supplementary data in S3. In addition, the present of OH

and CN group revealed the presence of phenolic and alkaloids compounds respectively [53]. Through this idea, it can be conclude that all the MPOTEF in both extracts having phenolic compound which supported by TPC result. Hence only DDF, SPF, VEL and VES in water extract with DDF in ethanolic extract was revealed containing alkaloids compound.

Table 5: The IR spectroscopic analysis of MPOTEF in water and ethanolic extract

Wave number (cm^{-1})														Type of bond	Functional group
Water extract							Ethanol extract								
NBF	DDF	GPF	SPF	ALF	VEL	VES	NBF	DDF	GPF	SPF	ALF	VEL	VES		
341	340	341	342	343	341	342	333	334	332	332	334	333	333	O-H, N-H(s)	Alcohol/Phenols
2.2	6.9	3.5	8.6	3.3	7.6	2.6	9.0	5.6	3.6	1.2	2.3	9.9	7.3		, Amine
292	292	292	292	292	292	292	292	292	293	297				C-H(s)	Alkanes
8.1	8.1	9.0	2.3	4.4	4.5	2.8	5.9	5.5	4.8	2.3					
								164	163	164		163	163	C=O(s), C=C(s),	Carbonyl
								2.2	7.1	9.1		6.7	7.1	N-H(b)	
163	160	162	162	164	161	161	164				163			C=C(s), N-H (b)	Alkenes, Amine
2.7	9.8	1.0	2.2	0.2	2.1	3.6	2.1				6.3				
	139	138	138	139	139	139				138				C-H(b)	Alkanes
	5.6	9.6	9.5	2.6	3.8	6.4				2.6					
136													135	N-O(s)	Nitro compound
6.9													5.2		
	126		127		125	125		126						C-N(s), C-O(s)	Amines, Acid
	9.4		8.3		6.2	8.9		6.6							
106	107	106	111	106	107	110	104	104	104	104	104	104	104	C-O (s)	Ester
5.9	2.1	5.5	5.0	9.7	6.7	8.5	3.6	2.9	0.1	3.4	8.1	6.2	5.0		
			668.											C-H(b), C-Cl(s)	Alkenes, Alkyl
			1												halide
618.		616.	618.		615.	615.	591.	602.	576.		644.	581.	603.	C-Cl(s)	Alkyl halide
2		3	5		2	3	9	0	9		2	1	6		
	533.	522.		544.			534.	542.	532.	530.	542.	544.	506.	C-Br(s)	Alkyl halide
	4	6		6			7	2	1	9	9	2	7		

CONCLUSION

The present study suggests that the MPOTEF possess significant antioxidant and moderate cytotoxic properties which were supported by the presence of alcohols, phenols, amine, alkanes, alkenes, alkyl halides, carbonyl, nitro compounds, acid, ether and ester detected by FTIR. PASS-predicted fern activity efficiently helps in selecting promising pharmaceutical leads with high accuracy and required antioxidant and radical scavenging. In future, these MPOTEF could be utilized as an alternative source of valuable drugs and therapeutic agents. Further studies are needed with this MPOTEF to isolate, characterize and elucidate the structure of the bioactive compounds to find out the possible mechanisms of antioxidant and cytotoxic properties.

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CONFLICT OF INTERESTS

The authors declare that they have no competing interests.

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