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The Comprehensive Study on Effective Concentration (EC_{50}) of Antioxidant Activities and Brine Shrimp Lethality of *Moringa oleifera*

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Moringa oleifera has gained substantial attention in industrial applications due to its remarkable antioxidant capabilities. Leveraging the potential of *Moringa oleifera* as a valuable and safe resource in pharmaceutical and nutraceutical applications is essential. This study aimed: (1) to determine the antioxidant activity of the ethanolic extracts and protein isolates of *Moringa oleifera* using FRAP, DPPH, and ABTS radical scavenging assays; and (2) to evaluate the cytotoxicity using the brine shrimp lethality assay. Results showed that fresh ethanolic extract (FEE) exhibited the highest radical scavenging activity (%RSA), followed by dry ethanolic extract (DEE), dry protein extract (DPE), and fresh protein extract (FPE) in DPPH and ABTS assays. All samples displayed significant FRAP activity. While FEE demonstrated the highest phenolic (109.59 mg GAE/g) and flavonoid contents (316.89 mg QE/g), FPE exhibited the highest toxicity among the samples. This study recommends 1.25 mg/mL of *Moringa oleifera* extract as an effective concentration for optimal antioxidant activity without exceeding toxicity thresholds. However, further comprehensive toxicological studies in animal models are advised to gain insights into the application of *Moringa oleifera* in the nutraceutical sectors. Identifying specific phenolic compounds through LC-MS and GC-MS is suggested to correlate with *in vivo* antioxidant activities and toxicity.

Keywords: Antioxidant; cytotoxicity; effective concentration; phenolic; protein

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Moringa oleifera has garnered significant attention for its nutraceutical and pharmaceutical potential owing to its diverse phytochemical composition present in all parts of the plant [1]. In 2020, the global market size surged to RM 31.329 billion, projected to reach RM 65.49 billion by 2028. The analysis of *Moringa oleifera* leaves has revealed the presence of alkaloids, glucosinolates, and crucial amino acids, contributing to its antimicrobial properties [2]. Furthermore, the plant is rich in essential minerals and vitamins like calcium, iron, magnesium, folic acid, vitamin B, and vitamin A [3]. Its potent antioxidant properties, attributed to phenolic compounds, along with ascorbic acid, carotenoids, and flavonoids, make it highly valuable in the realm of antioxidants both in young and mature leaves [2, 4]. Research has also highlighted the antioxidant and antimicrobial properties of *Moringa oleifera* from specific regions, such as southwestern states in Nigeria [5]. However, concerns exist over the potential cytotoxicity or cellular harm associated with *Moringa oleifera* despite its antioxidant and pharmacological properties. This study aims to provide a piece of comprehensive information on the effect of antioxidant concentration and the risk of cytotoxicity, given the increasing incorporation of *Moringa oleifera* in various products. Specifically, the research focuses on determining the total phenolic and flavonoid content in both protein isolates and ethanolic extracts from *Moringa oleifera*. Furthermore, it evaluates the effective concentrations of these extracts using various antioxidant assays (FRAP, DPPH, ABTS radical scavenging) while also assessing their cytotoxicity through brine shrimp lethality testing (BSLA). By gaining a deeper understanding of safe and effective antioxidant concentrations in *Moringa oleifera*, this study aims to align with sustainable development goals, particularly those related to good health and well-being (SDG 3) and zero hunger (SDG 2). Additionally, this study seeks to shed light on potential prooxidant effects from excessive antioxidant consumption, ensuring *Moringa oleifera* products offer benefits without compromising health.

EXPERIMENTAL

Chemical

1,1-dipehnyl-2-picrylhydrazyl (DPPH) (Merck, German), 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) (Merck, German), 2,3,5-

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Triphenyltetrazolium chloride (TPTZ) (Sigma-Aldrich, German), acetic acid (Systerm, Malaysia), aluminium chloride (Bendosen, Malaysia), ethanol 95% (HmbG, Malaysia) ferric chloride hexahydrate (Bendosen, Malaysia), Folin–Ciocalteu (Sigma-Aldrich, German), hydrochloric acid (HCL) [KSFE, Malaysia], sea salts (Double Swallow, Malaysia), sodium carbonate (Systerm, Malaysia), sodium acetate trihydrate (Labgene, India), sodium hydroxide (Bendosen, Malaysia), potassium persulfate (KSFE, Malaysia). All chemicals used are analytical grade.

Raw Materials

The mature *Moringa oleifera* leaves with consistent green colour were collected from Mr Moringa Shop Sdn Bhd at Kuala Nerus, Terengganu. The sample was packed in the ice box for 6 hours during transportation to UiTM Kampus Kuala Pilah and stored in the refrigerator (Panasonic, NR-B421T) at 4°C.

Sample Preparation

Dry Ethanolic Extraction

The samples were washed under running distilled water before dried using a drying oven for 3 hours at 60°C, crushed, and milled into powder form [6]. The sample was then soaked in 95% ethanol with a ratio of 5:1 for 48 hours with constant stirring. The extracts were filtered by using No. 1 Whatman Filter paper before concentrated using a rotary evaporator at 40°C and 123 mpas. The concentrated sample was stored at 4°C for further analyses.

Fresh Ethanolic Extraction

About 5 kg of fresh sample was submerged into 1 L of 95% ethanol for 48 hours with 18 hours of constant stirring. The extracts were filtered by using No. 1 Whatman Filtered paper concentrated in vacuum condition using a rotary evaporator at 40°C at 123 mpas. The concentrated sample was stored at 4°C for further analyses.

Dry Protein Isolate

The defatted sample was prepared by soaking the dried samples in hexane for 24 hours. Then, the defatted sample was evaporated to dry using a vacuum pump and stored at 20°C. The sample was air-dried for about 24 hours and milled into fine flour. The sample was dispersed and solubilized in 0.1 M NaOH at a ratio of 1:20 for 1 hour followed by centrifugation. The supernatant was treated with 0.1 M HCl or acetic acid until pH 5. The sample was left undisturbed for 8–12 hours in a chiller. Then, the sample was centrifuged to create protein precipitation and dried into drying pellets [1].

Fresh Protein Isolate

The fresh protein isolate was prepared according to the similar method as in dry protein isolate process.

Analysis of Antioxidant

Total Phenolic Content

The methodology was done following a previous study [7]. 10 mL of Folin–Ciocalteu reagent was mixed with 10 mL distilled water. Around 0.5 mL of samples were added into the 2.5 mL Folin–Ciocalteu reagent and incubated for 8 min in the dark. The solution was mixed with 2 mL of 7.5% freshly prepared sodium carbonate Na_2CO_3 and incubated for 1 hour. The absorbance was measured at 760 nm.

Total Flavonoid Content

10% of aluminium chloride ($AlCl_3$) was prepared by diluting 2.5 g of $AlCl_3$ with distilled water [7]. Then, 0.1 mL of 10% $AlCl_3$ was mixed with 2.8 mL of sample and incubated for 30 min. The absorbance was measured at 450 nm.

FRAP Reducing Assay

FRAP reagent was mixed with acetate buffer, TPTZ, and ferric chloride with a ratio of 10:1:1 [8]. Around 50 µL extract samples from different concentrations were placed with 1.5 mL of FRAP reagent. The solution was shaken thoroughly and incubated for about 10 minutes in dark condition at 37°C. The results of FRAP were measured at an absorbance of 593 nm once an intense base colour was displayed.

$$Frap\ Power: \frac{Absorbance\ (Sample) - Absorbance\ (Control)}{Absorbance\ (Highest) - Absorbance\ (Control)} \times 100$$

DPPH Free Radical Scavenging Assay

Around 4 mg of DPPH was added with 95% ethanol in a volumetric flask. Around 1 mL of ethanolic sample of each concentration was placed in a conical flask and mixed with 3 mL of prepared DPPH reagent. The solution was marked up to 10 mL using 95% ethanol, and placed in dark conditions for 30 min. The absorbance was measured at 517 nm spectrophotometrically [8]. The percentage of radical scavenging assay (%RSA) was calculated as the following equation:

$$\%RSA: \frac{Absorbance\ (Blank\ Control) - Absorbance\ (Sample - Blank\ corrected)}{Absorbance\ (Blank\ Control)} \times 100$$

ABTS-Radical Scavenging Assay

Around 7 mM ABTS reagent was mixed with potassium persulfate at a ratio of 1:1. The reagent was stored in the dark condition for about 12–16 hours and diluted with 95% ethanol (v/v) [9]. Accurately 5 µL from various concentrations was added into 4 mL diluted ABTS reagent solution. The solutions were shaken and protected from the light for about 7 minutes. The absorbance of this assay was measured at 734 nm [8]. The result was calculated according to the following equation:

$$\%RSA: \frac{Absorbance\ (Control) - Absorbance\ (Sample)}{Absorbance\ (Control)} \times 100$$

Determination of Protein Content

The protein content of dry and fresh samples was determined using the Kjeldahl method [10].

Brine Shrimp Lethality Assay (BSLA)

Egg nauplii were hatched at temperatures 28°C to 30°C with strong aeration conditions, in a continuous light regime. The seawater was prepared by adding sea salt into distilled water until the salinity reached 1.020 and the process was continued for about 48 hours for the egg to become nauplii. After hatching, around 10 nauplii were collected using a dropper and placed into a small container or beaker with 4 mL of sample from various concentrations and 40 mL artificial seawater. The assay was continued with calculating the surviving nauplii after 24 hours, and the lethality of exposure (LC_{50}) was determined [11]. The sample was run in triplicates (n=3) and Statistical Analysis System (9.1.4) software was used to analyse the results.

RESULTS AND DISCUSSION

Antioxidant Activities of *Moringa oleifera*

Figure 1 represents the TPC values for all four samples ranging from 58.07 to 90.89 mg/g. FEE exhibited the highest significant value of TPC (p<0.05), followed by DEE, FPE, and DPE. These results are consistent with the previous studies reported the presence of phenolic compounds like gallic acid, coumaric acid, ferulic acid, fumaric acid, chlorogenic acid, and caffeic acid in *Moringa oleifera* [12, 13].

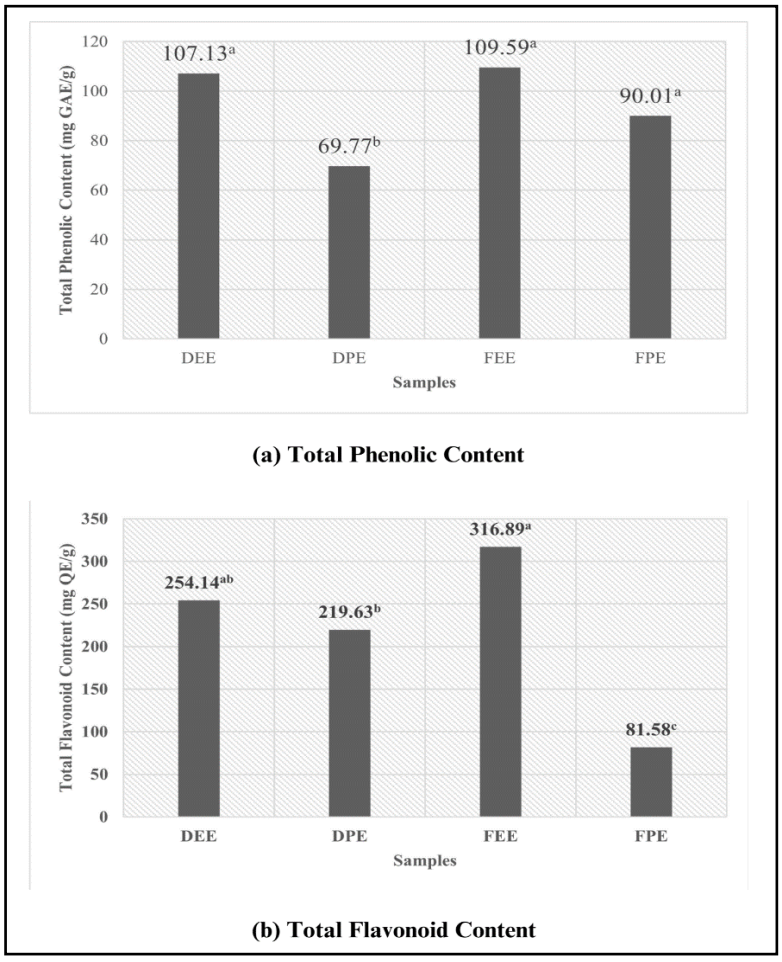


Figure 1. (a) Total phenolic content (TPC) in mg GAE/g and (b) total flavonoid content (TFC) in mg QE/g of each sample.

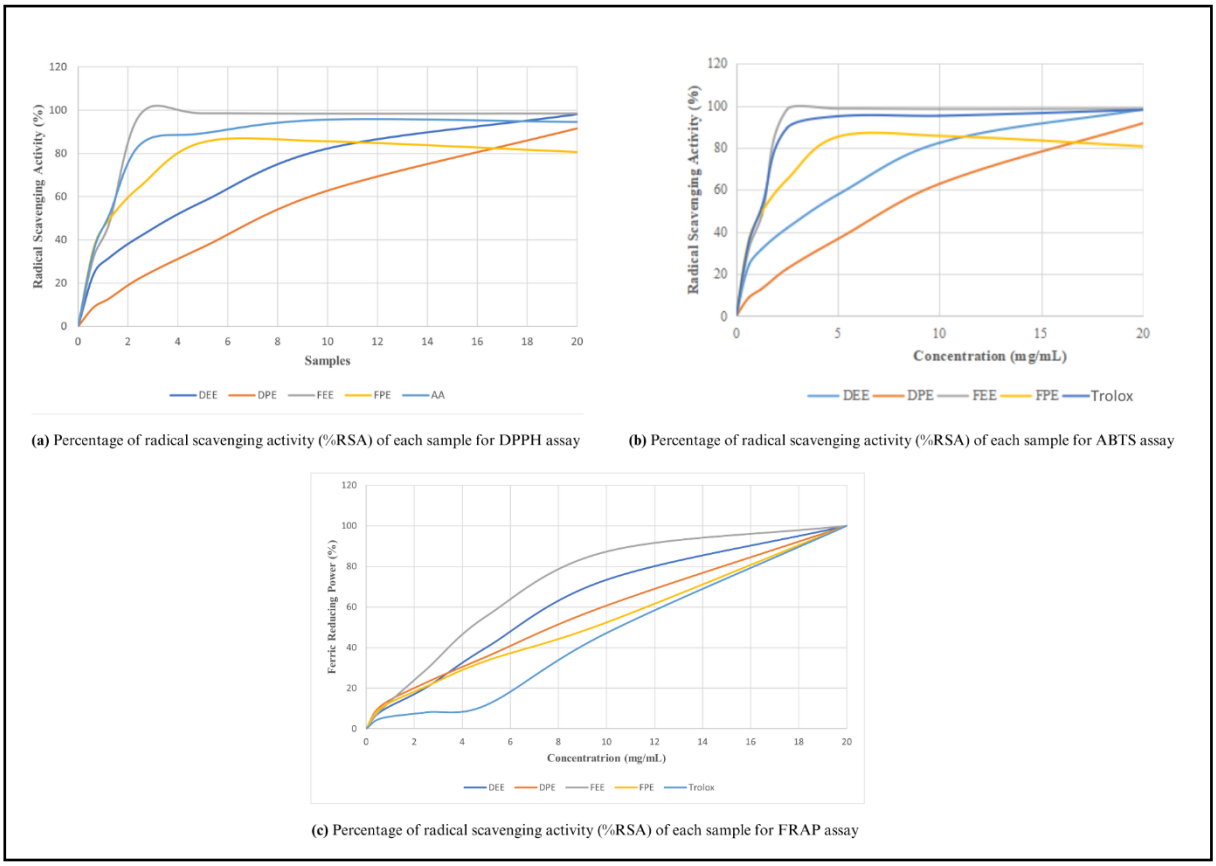
Note: * DEE is dry ethanolic extract, DPE is dry protein extract, FEE is fresh ethanolic extract, and FPE is fresh protein extract of *Moringa oleifera.*

However, another study reported the TPC values of *Moringa oleifera* from methanol extract ranging from 3.0 to 30.0 mg GAE/g [14]. The disparity in TPC values can be due to differences in the extraction method, the type of solvent utilized, and the preparation techniques applied to the sample.

The total flavonoid content (TFC) is typically expressed in Quercetin Equivalent (QE) per 100 grams of the sample or extract weight in grams (mg QE/100g), as illustrated in Figure 1. Among the samples, FEE displayed the significantly (p<0.05) highest flavonoid content, followed by DEE, DPE, and FPE. The TFC values ranged from 316.893 (FEE) to 81.576 (FPE) mg QE/g. Based on the overall dataset, each sample exhibited a significant difference (p<0.05) from one another. Numerous studies have reported the TFC values in different extracts of *Moringa oleifera* including leaves, stems, and roots, with the values ranging from 500 to 2400 mg QE/mg dry weight (DW). Lower values of 600 mg QE/mg DW and 500 mg QE/g DW were observed in the extracts of roots and stems, respectively [15]. Additionally, another study reported lower TFC readings for *Moringa oleifera* leaves and stem, and claimed that the highest flavonoid content was found in Moringa leaves (113.95 mg QE/g dry extract), followed by the stem (66.16 mg QE/g dry extract) [16].

Research on flavonoid profiling, revealed the presence of consistent flavonoids, including quercetin-3-O-glucoside, quercetin-3-O-(6″-malonyl) glucoside, quercetin-3-O-(X″-malonyl)glucoside, kaempferol-3-O-glucoside, and kaempferol-3-O-malonylglucoside [12]. These findings aligned with another study that reported the primary flavonoids in *Moringa oleifera* consist of derivatives of kaempferol, myricetin, and quercetin [17]. Additionally, glycosylated flavonoids resulting from the condensation of kaempferol with rhamnose and glucose, specifically quercetin-3-O-glucoside-7-O-rhamnoside and methyl-O-quercetin-malonylglucoside, were detected. Notably, the leaves of *Moringa oleifera* exhibited the presence of isorhamnetin-rhamnosyl-glucoside and a diglucoside, possibly composed of quercetin bound to two rhamnose units or kaempferol condensed with a rhamnosyl-glucoside.

Figure 2 shows antioxidant activities of *Moringa oleifera* measured by FRAP assay, DPPH and ABTS radical scavenging assays. The %RSA of DPPH assays for all samples were in the range of 0% to 98.50% as in Figure 2(c). The initial concentration of standard ascorbic acid (AA) was 0 mg/mL and reached stagnant at 5 mg/mL. At the stagnant phase, the percentage of radical scavenging activity (%RSA) was measured to be 94.95%.



(a) Percentage of radical scavenging activity (%RSA) of each sample for DPPH assay

(b) Percentage of radical scavenging activity (%RSA) of each sample for ABTS assay

(c) Percentage of radical scavenging activity (%RSA) of each sample for FRAP assay

Figure 2. Antioxidant activities of *Moringa oleifera*.

Note: (a) DPPH, (b) ABTS, and (c) FRAP assays. DEE is dry ethanolic extract, DPE is dry protein extract, FEE is fresh ethanolic extract, FPE is fresh protein extract of *Moringa oleifera* and Trolox is standard, AA is ascorbic acid standard.

Meanwhile, the reading of %RSA for FEE was observed to become stagnant starting at 2.5 mg/mL in which the %RSA was 98.19%, and the maximum %RSA of FEE was exhibited at 20 mg/mL with the value of 98.50%. This observation indicated that further addition of extract would not exhibit any antioxidative effect. This result also implied the maximum concentration of the extract that can be applied to achieve maximum output. The %RSA for DPE did increase gradually until the last reading of %RSA at 91.6% which was at 20 mg/mL. On the other hand, DEE showed the same trend as DPE although the final reading of %RSA was at 98.17%. The percentage radical scavenging activity of FPE increased significantly at 5.0 mg/mL with a slightly shallow line. This finding indicated the decrement in antioxidant activity, and the ability of the sample to scavenge or neutralize the free radicals has decreased.

ABTS antioxidant assay showed the same trend of %RSA from DPPH as shown in Figure 2 (a). All four samples and standards increased by more than 90%. Nevertheless, Trolox (5 mg/mL), DEE (1.25 mg/mL), and FEE (1.25 mg/mL) were at respective concentrations. Therefore, the addition of a sample would not cause any increase in antioxidant activity. DPE increased gradually with the final observation of %RSA at 98.06%. The same trend can be observed in FPE, as it increased gradually before slowly declining at a concentration of 2.0 mg/mL from 92.57% to 84.21%. The decrease in %RSA of FPE can be attributed to the reduction of the radical scavenging activity to neutralize the free radicals.

FRAP assay is bound to have a different graph compared to DPPH (Figure 2(a)) and ABTS (Figure 2(b)) due to differences in the antioxidative mechanism of the assays. Based on Figure 2, 100% ferric-reducing power was achieved for all the samples at a concentration of 20 mg/mL. Nevertheless, the ethanolic sample was proved to produce a higher output of FRAP power as this can be evidenced through the readings of FEE and DEE, as both ethanolic samples passed 50% of FRAP power even at a concentration of 10 mg/mL. These findings were in line with the previous studies which stated that alcoholic extracts produce and contribute to higher antioxidant compounds that can be retained [18, 19].

Half-Maximal Effective Concentration (EC_{50})

Median effective concentration at 50% (EC_{50}) or half-maximal effective concentration is a measurement of a compound concentration that is required to produce 50% of biological reaction and causes a reaction. Therefore, it is calculated as midway between the baseline and the maximum effective concentrations. EC_{50} is applied to measure the effectiveness of an antioxidant in scavenging free radicals or its reducing capability. The lower the EC_{50} value, the more effective the antioxidant at scavenging free radicals, indicating a smaller amount of the antioxidant is required to reduce 50% of the free radicals. Table 1 displays the experimental data on EC_{50} of DPPH, ABTS, and FRAP for standard (Trolox and Ascorbic Acid), DEE, DPE, FEE, and FPE. In DPPH assay, standards ascorbic acid (AA) exhibited the significant lowest value ($p<0.05$) of EC_{50}, which was $0.05^{d} \pm 0.98$ mg/mL followed by FEE ($0.63^{c} \pm 0.08 \times 10^{-2}$ mg/mL), FPE ($0.63^{c} \pm 0.012$ mg/mL), DEE ($8.24^{b} \pm 0.059$ mg/mL), and lastly DPE ($9.79^{a} \pm 0.059$ mg/mL). In this assay, the data ranging from 0.05 to 9.79 mg/mL with both FEE and FPE did not show any significant difference. In contrast, both DEE and DPE samples showed significant differences. In terms of ABTS assay, FEE displayed the significant lowest value ($p<0.05$), with the value of $0.62^{d} \pm 0.002$ mg/mL, followed by DEE ($0.64^{d} \pm 0.08 \times 10^{-2}$ mg/mL), standard Trolox ($1.02^{c} \pm 0.030$ mg/mL), FPE ($2.07^{b} \pm 0.02$ mg/mL), and lastly DPE ($5.51^{a} \pm 0.16$ mg/mL). This result implied that FEE and DEE did not show any significant difference. In contrast, standard Trolox, FPE, and DPE did show significant differences between each other. Apparently, utilizing FRAP assay demonstrated the range of EC_{50} to be from 1.01504 to 8.87422 with the lowest significant value ($p<0.05$) of EC_{50} in standard Trolox ($1.02^{d} \pm 0.05$), followed by DEE ($8.87^{c} \pm 0.002$), FEE ($9.34^{b} \pm 0.17$), DPE ($9.64^{a} \pm 0.08 \times 10^{-2}$) and FPE ($9.72^{a} \pm 0.02$). In terms of samples ranking, FEE sample produced the lowest value of EC_{50} ($p<0.05$) by using ABTS, followed by DPPH assays. Both fresh samples (FEE and FPE) showed the same trend value of EC_{50} as DPPH displayed the lowest value out of all assays. This can be further explained in terms of the mechanism for each assay. The mechanism of each assay will be explained in further discussion.

Previous studies have suggested that the EC_{50} of ethanolic extract *Moringa oleifera* can be around 0.024 to 0.043 mg/mL [20, 21]. However, this range did not appear to be the case of this study as this analysis produced a different value of EC_{50}. The main reason of the discrepancies in this value is due to the different processing methods, genomic, post and preharvest, geographical location, and ecological factors. These factors are crucial in determining the antioxidant potential of plants [22]. Since EC_{50} is the reduction of 50% of DPPH concentration [23], the value obtained is closely related to %RSA, in which the %RSA is based on the plant itself. However, the study on the EC_{50} of leaf protein isolates is still lacking therefore limiting our discussion on the current findings.

Protein Content by Kjeldahl Method

Based on the present study, fresh protein extract (FPE) of *Moringa oleifera* contained higher value (19.76±0.54) compared to dry protein extract (DPE) (54.06±0.21). This is due to DPE undergone several heating treatments to ensure the sample was dry for further analysis. Prior discoveries have stated that *Moringa oleifera* protein content ranged from 10% – 56% [24, 25]. The protein composition of *Moringa oleifera* leaves encompasses various fractions,

including glutelin, albumin, prolamin, globulin, and insoluble proteins, such as coagulation factor II and prothrombin [21].

Correlation of Antioxidant Assays (EC50) of FRAP, and DPPH, ABTS Assays

Table 2 demonstrates the correlation of antioxidant assays for DPPH, ABTS, and FRAP with the samples consisting of FPE and DPE. The results that were analysed using Statistical Analysis System (SAS) version 9.1.4 displayed a negative correlation for all analyses except for ABTS and FRAP assays. The highest correlation could be found from the ABTS-FRAP correlation (R^2 = 0.9631) and the lowest was DPPH-ABTS (R^2 = -0.99987). The main reason FRAP and ABTS have a high positive correlation is due to the mechanism of assays. FRAP method consists of antioxidants that are compared according to their capacity to donate an electron to ferrous (Fe^{2+}) ions to reduce ferric (Fe^{3+}) to ferrous (Fe^{2+}) ions. This mechanism is classified as SET-mechanism and ABTS assay is a photometric assay that relies on the ability of antioxidant compounds to reduce a widely recognized unstable radical, leading to a measurable response [26]. ABTS can apply both HAT and SET mechanisms. However, ABTS assays mainly follow the SET-mechanism [27]. DPPH-ABTS and DPPH-FRAP on the other hand showed negative correlations due to the nature of the DPPH assay which is a radical-HAT-based method that differentiates itself from the other two assays in which DPPH applied the donation of electron or hydrogen radical to stabilize the free radical of DPPH [28].

Table 1. EC_{50} values of four different treatments of *Moringa oleifera.*

Samples	EC_{50}		
	DPPH	**ABTS**	**FRAP**
Standard ascorbic acid (AA)	$0.05^{d} \pm 0.98$	-	-
Standard Trolox (TRX)	-	$1.02^{c} \pm 0.030$	$1.02^{d} \pm 0.05$
Dry *Moringa oleifera* ethanolic extract (DEE)	$8.24^{b} \pm 0.059$	$0.64^{d} \pm 0.08 \times 10^{-2}$	$8.87^{c} \pm 0.002$
Dry *Moringa oleifera* protein extract (DPE)	$9.79^{a} \pm 0.059$	$5.51^{a} \pm 0.16$	$9.64^{a} \pm 0.08 \times 10^{-2}$
Fresh *Moringa oleifera* ethanolic extract (FEE)	$0.63^{c} \pm 0.08 \times 10^{2}$	$0.62^{d} \pm 0.002$	$9.34^{b} \pm 0.17$
Fresh *Moringa oleifera* protein extract (FPE)	$0.63^{c} \pm 0.012$	$2.07^{b} \pm 0.02$	$9.72^{a} \pm 0.02$

Table 2. Correlation between EC_{50} of the antioxidant activities and different extraction treatment of *Moringa oleifera*

(a) Correlation between EC_{50} of the antioxidant activities and protein extract (DPE and FPE)

Pearson Correlation Coefficient, N = 6			
	DPPH	**ABTS**	**FRAP**
DPPH	1.00000	-0.99987	-0.96223
ABTS	-0.99987	1.00000	0.96131
FRAP	-0.96223	0.96131	1.00000

Note: Dried protein extract denotes by DPE, and fresh protein extract denotes by FPE

(b) Correlation between EC_{50} of the antioxidant activities and ethanolic extract (DEE and FEE)

Pearson Correlation Coefficient, N = 6			
	DPPH	**ABTS**	**FRAP**
DPPH	1.00000	0.99950	-0.93438
ABTS	0.99950	1.00000	-0.94023
FRAP	-0.93438	-0.94023	1.00000

Note: Dried ethanolic extract denotes by DEE and fresh ethanolic extract denotes by FEE

Ethanolic extract (DEE and FEE) almost have identical trends as protein samples, with the exception that DPPH-ABTS have a strong positive correlation (R^2 = 0.99950). The possible reason to the deviations is due to the mechanisms itself. The DPPH assay is known to apply the HAT mechanism, meanwhile ABTS assay can apply both HAT/SET mechanisms. Both assays possessed the same mechanism and ability that scavenge or neutralize the free radicals. However, comparing both tables, the most significant effect that causes deviations is the nature of the samples. A previous study stated that FRAP, DPPH, and ABTS assays are most likely to be influenced by the structural composition and origin of the sample itself [28]. There are some findings by the same authors that reported on all positive correlations for FRAP, DPPH, and ABTS assays for guava fruit extracts, and aqueous extracts came from lentils and beans. Therefore, deep study and understanding on the properties of samples and assays are emphasized to ensure an appropriate match.

Toxicity and LC_{50} by Brine Shrimp Lethality Assay (BSLA)

Based on Table 3, brine shrimp cultivated for 24 hours at 20 mg/mL exhibited the highest mortality rate with the average mortality of four sampl-0 es were 44.44% followed by 5 mg/mL. The rest ppm did not display any kind of mortality towards the brine shrimp (*Artemia salina*). At 1 hour, there was no mortality recorded. The present results highlighted the detrimental effects that the *Moringa oleifera* concentration has on the mortality rate of brine shrimp. The study of EC_{50} for protein isolate of *Moringa oleifera* is still lacking hence limiting the discussion on the current findings.

The concept of LC_{50} explains that the smaller the value, the bigger or the higher the toxicity of the extract. The lowest value of LC_{50} was found in the DPE sample with the value of 17.46 ± 0.011(Table 4). The highest LC_{50} was observed in DEE at 6h with the value of 96.09 ± 0.012. There was almost no difference between the DPE and FPE samples, as the data was almost identical. In terms of toxicity, FPE demonstrated the highest toxicity. In this case, the protein sample was much more toxic compared to ethanolic extract due to the presence of insoluble protein (antinutritional factor). Antinutritional factors a biological constituents found in food that can hinder the absorption and utilization of nutrients, resulting in compromised gastrointestinal functions and metabolic performance [29]. This antinutritional factor is included with lectins which are a class of proteins that exhibit specific binding to carbohydrates, which consequently can exert toxic effects on certain organisms, such as brine shrimp (*Artemia salina*).

Table 3. The result of cytotoxic activity of dry *Moringa oleifera* ethanolic extract and protein extract on brine shrimp (*Artemia salina*).

Sample	Concentration (mg/mL)	Log C	Mortality rate of brine shrimp (%)		
			1h	6h	24h
DEE	0.039	-1.4089	0	0	0
	0.156	-0.80688	0	0	0
	0.625	-0.20412	0	0	0
	1.250	0.09691	0	0	0
	5.000	0.69897	0	11.11	44.44
	20.000	1.30103	0	44.44	44.44
DPE	0.039	-1.4089	0	0	0
	0.156	-0.80688	0	0	0
	0.625	-0.20412	0	0	0
	1.250	0.09691	0	0	0
	5.000	0.69897	0	0	11.11
	20.000	1.30103	0	20	44.44
FEE	0.039	-1.4089	0	0	0
	0.156	-0.80688	0	0	0
	0.625	-0.20412	0	0	0
	1.250	0.09691	0	0	0
	5.000	0.69897	0	11.11	22.22
	20.000	1.30103	0	11.11	44.44
FPE	0.039	-1.4089	0	0	0
	0.156	-0.80688	0	0	0
	0.625	-0.20412	0	0	0
	1.250	0.09691	0	0	0
	5.000	0.69897	0	0	22.22
	20.000	1.30103	0	30	55.55

Note: DEE is dry ethanolic extract, DPE is dry protein extract, FEE is fresh ethanolic extract, and FPE is fresh protein extract of *Moringa oleifera.*

Table 4. LC_{50} values of DEE, DPE, FEE, and FPE of *Moringa oleifera* based on time intervals of 1h, 6h, and 24h.

Sample	LC_{50}		
	1h	**6h**	**24h**
Dry *Moringa oleifera* ethanolic extract (DEE)	-	96.09 ± 0.012	24.02 ± 0.006
Dry *Moringa oleifera* protein extract (DPE)	-	35.58 ± 0.003	17.46 ± 0.011
Fresh *Moringa oleifera* ethanolic extract (FEE)	-	76.89 ± 0.058	21.34± 0.01
Fresh *Moringa oleifera* protein extract (FPE)	-	35.59 ± 0.006	17.45 ± 0.01

Note: DEE is dry ethanolic extract, DPE is dry protein extract, FEE is fresh ethanolic extract, and FPE is fresh protein extract of *Moringa oleifera.* 6h is 6 hours and 24 h is 24 hours.

Table 5: The correlation table between EC_{50} and LC_{50} for different categories and assays for DEE, DPE, FEE, and FPE.

Pearson Correlation Coefficients, N = 4					
	LC_{50} (24h)	LC_{50} (6h)	DPPH	ABTS	FRAP
LC50 (24h)	1.00000	0.99620	0.13168	0.69878	-0.98053
LC50 (6h)	0.99620	1.00000	0.07502	0.63835	-0.96155
DPPH	0.13168	0.07502	1.00000	0.31716	-0.30895
ABTS	0.69878	0.63835	0.31716	1.00000	-0.77737
FRAP	-0.98053	-0.96155	-0.30895	-0.77737	1.00000

This finding is consistent with the previous research that reported the cytotoxic effects on the brine shrimp by using *Moringa oleifera* leaves [30]. Furthermore, another research has proved that the lectins from *Moringa oleifera* contain toxicity causing larvicidal of *Aedes aegypti* larvae [31]. The previous studies on *Moringa oleifera* proved that LC_{50} ranged from 4.3×10^{-4} – 1.18×10^{-3} mg/mL [32]. Precisely, leaves extract had the highest value of LC_{50} at 8.12×10^{-3} mg/mL due to the presence of antinutritional compound [33].

Correlation of Antioxidant Assays (EC_{50}) of DPPH, ABTS, and FRAP Assays with Cytotoxicity (LC_{50}) Test

According to Table 5, fractions derived from the *Moringa oleifera* leaves extract exhibited cytotoxic effects on the brine shrimp nauplii especially LC_{50} of ABTS assay. The previous result obtained explains the possibility of prooxidants that exist within the protein sample. The existence of prooxidants can be the key factor in the cytotoxicity of brine shrimp nauplii. DPPH and FRAP assays did not exhibit a strong correlation with LC_{50}. The main reason is due to the mechanism of the assays and the properties of the sample itself. This condition can be explained through the mechanism of FRAP assay, as the EC_{50} value of FRAP assay has a negative correlation with LC_{50} as this assay is constrained due to the presence of hydrophilic substances [34]. *Moringa oleifera* is abundant with hydrophobic compounds, hence, FRAP assay is not suitable for certain samples [34]. DPPH is thus produced a weak correlation for both LC_{50} at 6h and 24h. Although both DPPH and ABTS assays followed a similar mechanism, they differ in the chemical properties of the radicals they target and the specific testing conditions. These distinctions can affect how the tested substances interact with the radicals, resulting in varying assessments of antioxidant effectiveness. As a result, DPPH assay may not yield the same prooxidant results as ABTS assay, contributing to weak correlations between DPPH results and LC_{50} values.

CONCLUSION

In conclusion, this paper highlights the optimal concentration of *Moringa oleifera* leaf extract and protein isolate through various antioxidant assays, including FRAP, DPPH, ABTS radical scavenging, as well as measuring total flavonoid content (TFC) and total phenolic content (TPC). Additionally,

the study assessed the cytotoxicity of *Moringa oleifera* ethanolic extract and protein isolate using the brine shrimp lethality test. The results showed that both fresh ethanolic extract (FEE) and protein isolate samples demonstrated significant antioxidant activity, with FEE demonstrated the lowest EC_{50} values for DPPH and ABTS assays. The use of ethanol in the extraction process increased the effectiveness of extracting antioxidant compounds from *Moringa oleifera*. The analysis also revealed a higher concentration of flavonoids than phenolic compounds in *Moringa oleifera* from the specified source. Protein content analysis indicated higher levels in the FPE samples compared to DPE. However, the cytotoxicity test highlighted the need for caution in using high concentrations of *Moringa oleifera* extracts, as higher concentrations and longer exposure times led to increased mortality rates in brine shrimp. The FPE sample exhibited the highest toxicity based on the LC_{50} values. The present study proposes further research to elucidate the specific roles of flavonoids and phenolic compounds in the antioxidant activity and cytotoxic effects of *Moringa oleifera.* Recommendations included employing GC-MS and HPLC for compound specification, conducting functional analysis of enzymic activities, evaluating Hydrogen Peroxide scavenging activity assay for *in vivo* assessment of antioxidant activity, and utilizing Sprague Dawley mice for a more comprehensive analysis of *Moringa oleifera* toxicity.

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