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RESEARCH ARTICLE

In vitro Antioxidant Capacity of *Peperomia pellucida* (L.) Kunth Plant from two different locations in Malaysia using different Solvents Extraction

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ABSTRACT:
The study was performed to investigate the antioxidant capacities of *Peperomia pellucida* (L.) collected from two different locations using different solvents extraction. Antioxidant capacity was analyzed by 1, 1-Diphenyl-2-picryl hydrazyl (DPPH), *β*-carotene bleaching and oxygen radical absorbance capacity (ORAC) assays. The ability to scavenge free radical in DPPH assay showed that methanol extract of Penang (ground) sample depicted the highest value of 1108 ± 5.568 mg/ml compared to the other samples. In *β*-carotene bleaching assay, the ethyl acetate extract of Penang (ground) sample offered the highest percentage of scavenging activity from free radical (86.111%). In ORAC assay, methanol extract of Penang (ground) sample showed the highest value 397.691 µmol of Trolox equivalent/µl. Results between two locations showed no significant difference as compared to the samples from Selangor except for DPPH scavenging assay which depicted a highly significant difference ($p<0.05$) in Penang. Meanwhile, there was a significant difference ($p<0.05$) between hexane, chloroform, ethyl acetate and methanol extract of DPPH scavenging, *β*-carotene bleaching and ORAC assay. Findings showed that ethyl acetate and methanol extracts exhibited a good solvent for antioxidant capacity of *P. pellucida*. Thus, *P. pellucida* could be a potential source of antioxidant substance.

KEYWORDS: *Peperomia pellucida*, Antioxidant Capacity, DPPH, *β*-carotene bleaching, ORAC.

INTRODUCTION:
The priority and concern towards having a healthy lifestyle has increased in recent years. Medicinal plant is acceptable as a primary health care due to the minimal side effects and beneficial to the human body[1]. The chemical compounds in plants such as alkaloids, flavonoids, steroids, terpenoids, carotenoids, tannins and glycosides are the most important compounds[2] which are also responsible for the bioactivities of the plants such as antioxidant, antifungal, antimicrobial[3], antiviral, anti-inflammatory, antitumor, analgesic and antimalarial[4]. Antioxidant is a synthetic or natural substance which slows down the harmful effects of free radicals[5].

Free radical is a molecular species which is able to exist independently and contains an unpaired electron in an atomic orbital[6] and can form toxic wastes and dangerous to human body[7]. In order to combat free radical, human body is struggling to obtain antioxidants whereby the consumption of medicinal plants is one of the alternative ways for antioxidants sources.

Extraction is an important step to discover the active compounds in the plant materials[8]. Nowadays, extraction was done in various methods as there is no single and standard method of extraction to isolate, identify and use of phenolic compounds of the plant[9]. Commonly, solvent extraction was used by many researchers for plant extraction because it is simple, effective and large applicability[10] especially to extract plant antioxidant compounds[11]. Different types of solvents with varied polarities, the time taken and temperature during plant extraction as well as solvent to solvent ratio used will also affect the extract yield[10]. As selected solvents greatly affected the extract yield, many solvents were used for plant extraction.

P. pellucida (L.) is one of the well-known medicinal plants worldwide, belongs to the family of Piperaceae[12]. This plant is easily found in Malaysia especially in a moist habitat and is believed to confer antioxidant properties. Several studies on *P. pellucida* focussed on the plant extract as immunostimulator[13], anticancer[14], antimicrobial[14,15], anti-inflammatory[16], antibacterial[17], nutritional and mineral composition study[18,19], toxicity studies[20,21], antipyretic study[22,23], analgesic activity[24] and chemical compound studies[25]. The presence of chemical compounds in *P. pellucida* such as the phenolic compounds[26], flavonoids[27,28], alkaloids, tannins and saponins[29] are responsible for the antioxidant properties of this plant and this finding has become the main reason for further antioxidant investigation of this plant.

Generally, the origin of the plant sample will affect the plant growth as well as the plant's chemical compounds[30]. Besides,[31] also claimed that the physical environmental factors for plant growth such as rainfall, climate and altitude could also affect the quality of the plant's extract even the extract is produced from the same country. Meanwhile, according to[32], soil nutrients and fertilizers can affect the plant yield and the quality of the plants. Furthermore, the soil organic matter can also alter the pH of the surrounding soil[33] in which the soil pH is important for the availability of nutrient in plant[34, 35]. Thus, the selection of different locations and sources are one of the important factors that have been considered before the plant extraction. The purpose of this study is to investigate the antioxidant capacities of *Peperomia pellucida* (L.) collected from two different locations which are Selangor and Penang area by *in vitro* antioxidant study using different solvents extraction (hexane, chloroform, ethyl acetate and methanol).

MATERIAL AND METHODS:

Research Sample:

A species of medicinal plant used in this study is *Peperomia pellucida* (L.), which belongs to the family of Piperaceae. All samples were collected from different locations of nursery around Selangor and Penang area with several batches. The collected plant samples were separated between the ground and the flower pot. Plant sample was identified by Associate Prof. Dr. Fatimah Mohamed from Biology Department, Universiti Pendidikan Sultan Idris and deposited to the Herbarium of Universiti Pendidikan Sultan Idris with herbarium number AS001 and AS003.

Preparation of solvent extraction:

P. pellucida was collected from two different locations which were Penang and Selangor, Malaysia. In each locality, the plant was collected separately; from the ground and from the flower pot. The whole *P. pellucida* plant included leaves, stems and roots were washed thoroughly with tap water to remove dirt and the samples were air dried for 21-25 days under room temperature. The dried whole plants were grinded into coarse powder form and stored in an air tight container at 20ºC before further experiments. The samples then were extracted sequentially using solvents of increasing polarity from hexane, chloroform, ethyl acetate and methanol. For each solvent, the powdered samples were soaked for 72 hours[36]. Filtration was done using filter papers and filtrate was then evaporated using rotary evaporator under reduced pressure[37,36]. The crude extract was stored in a tight container in room temperature until further experiment.

In vitro Antioxidant Assays:

The antioxidant activities of *P. pellucida* plant were determined using three different types of antioxidant assays which were DPPH, *β*-carotene bleaching and Oxygen Radical Absorbance Capacity (ORAC) assays.

DPPH Scavenging Assay:

Five mg of 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Merck Millipore, EMD Millipore Corp., Billerica, MA USA) was dissolved in methanol as in previous method[37,36]. The extracted sample was diluted into various concentrations and prepared in 96-well 230 plates in triplicates and followed by the addition of five microliters of methanolic DPPH solution with final concentration of 300µM of DPPH. After 30 minutes of incubation in room temperature, the optical density (OD) of the reaction mixture was read at 517nm using microplate reader. The percentage inhibition was calculated by the following formula:

$$\% \text{ Inhibition} = \frac{\text{1-OD (DPPH + sample)}}{\text{OD (DPPH)}} \times 100$$

The half maximal inhibitory concentration (IC50) value was obtained by plotting the graph DPPH scavenging percentage against the concentration of the sample extract.

β-carotene Bleaching Assay:

The method used in *β*-carotene bleaching assay was done as in[36]. Briefly, 210µl of *β*-carotene solution (1 mg ml-1 in chloroform) was transferred into a round bottom flask containing five microliters linoleic acid and 42µl Tween 20. After that, the chloroform was removed by rotary evaporator at 40ºC. Emulsion was formed by the addition of distilled water (10ml) to the round bottom flask with vigorous shaking. An aliquot of 200µl from the emulsion were then added into each of the 96 well microplates which contain 50µl of samples (1 mg ml-1). The absorbance was read at 470nm. For this assay, butylated hydroxytoluene (BHT) and α-tocopherol were used as standards reference. Reading of all samples were done at 0 hour (t = 0) and after 2 hours (t = 2) of incubation at 50ºC in the dark. The antioxidant activity (AA) was calculated according to the formula given

below:

$$AA\% = 1 - \frac{At=0 - At=2}{Ac=0 - A\ c=2} \times 100$$

Where At=0 and At=2 is the absorbance of the test samples measured at 0 hour and 2 hours, respectively, Ac=0 and Ac=2 is the absorbance of control (*β*-carotene-containing emulsion and methanol instead of test samples) measured at 0 hour and 2 hours, respectively.

Oxygen Radical Absorbance Capacity (ORAC) Assay:

The method used in ORAC assay was as in previous study[38,39,37]. In this assay, the FLUOstar OPTIMA microplate fluorescence reader was used with an excitation wavelength of 485 nm and an emission wavelength of 520 nm. ORAC assay used fluorescein (10nM) that was prepared earlier in 75mM phosphate buffer (pH 7.4) and stored at 4°C in a dark condition. After that, the preparation of 2,2'-Azobis (2-amidino-propane) dihydrochloride (AAPH) of 153mM and 6-hydroxy-2,5,7,8-tetra- methylchroman-2- carboxylic acid (Trolox) of 2mM in 75mM phosphate buffer (pH 7.4) were done. Trolox standard was diluted in the phosphate buffer to give 1.5µM to 50µM of working solutions. To start the reaction, 150µL of fluorescein was added into the 96-well plates and followed by 25µL of trolox, buffer (blank), or sample that were carried out in the same run. The mixture was preincubated for 15 min at 37oC directly in a microplate reader. Then, AAPH (25µL) was injected to the mixture via injector. Fluorescence readings were taken at every minute for 80 minutes. ORAC values were calculated based on net area under the curve (AUC) obtained by subtracting the AUC of the blank from that of a sample and compared to Trolox standards curve. The antioxidant capacity (ORAC) related to trolox is calculated as:

$$\text{ORAC value} = \frac{\text{AUCsample-AUCblank}}{\text{AUCTrolox-AUCblank)}\ [\text{trolox}]\ \text{dilution factor}}$$

Statistical Analysis:

All results of three *in vitro* antioxidant assay of DPPH, *B*-carotene bleaching and Oxygen Radical Absorbance Capacity (ORAC) assays were analysed and performed using SPSS version 20 and independent t-test or one way ANOVA to compare the results between different locations, sources of samples and solvents used to determine the antioxidant capacity of *P. pellucida* plant extract.

RESULTS AND DISCUSSIONS:

***In vitro* antioxidant capacity:**

The *in vitro* antioxidant capacity was analyzed spectrophotometrically by 2,2-diphenyl-1-picrylhydrazyl (DPPH), *β*-carotene bleaching and oxygen radical absorbance capacity (ORAC) assays. The whole results of three antioxidant assays are summarized in Table 1.

Table 1 : The result of *in vitro* antioxidant assays in *P. pellucida* plant from different locations

Samples	DPPH assay (IC_{50} value, mg/ml)	*B*-carotene assay (% inhibition)	ORAC assay (µmol TE/25 µl)
Std a	Ascorbic acid:20.210±0.441	α-tocopherol:96.710	-
Std b	α-tocopherol: 14.710±0.062	BHT:81.990	-
PG-1	2744±6.557	41.101	57.960
PF-1	2011±8.718	35.194	34.911
SG-1	1816±15.524	67.952	35.161
SF-1	1732±12.288	63.890	56.142
PG-2	2875±12.288	56.331	62.384
PF-2	1524±11.358	49.770	35.852
SG-2	1798±8.888	31.613	24.263
SF-2	1589±7.000	32.582	41.662
PG-3	1380±3.606	86.111*	368.470
PF-3	1391±7.211	75.101	304.080
SG-3	1279±7.810	69.260	257.120
SF-3	1255±7.000	63.890	319.23
PG-4	1241±5.292	62.072	397.691*
PF-4	1432±4.583	52.943	382.400
SG-4	1108±5.568*	67.822	365.690
SF-4	1245±5.000	71.044	232.881

*represents the highest antioxidant activity in each antioxidant assay
DPPH assay values are expressed as mean±SD of 3 readings
Note, Std a and Std b = Standard; PG = Penang ground; PF = Penang flower pot; SG = Selangor ground; SF = Selangor flower pot; 1 = Hexane extract; 2 = Chloroform extract; 3 = Ethyl acetate extract; 4 = Methanol extract.

The DPPH assay result is defined as the amount of antioxidant needed to decrease the initial DPPH radical concentration by 50% (IC_{50}) in 30 minutes, thus the lowest IC_{50} values represented the highest antioxidant capacity[40]. In this study, ascorbic acid and alpha tocopherol have been used as standards in DPPH assay. The hexane extract of the whole plant sample from Selangor-flower pot (SF-1) showed the best antioxidant activity to inhibit 50% of DPPH with the value of 1732±12.288 mg/ml followed by the samples from Selangor-ground (SG-1), Penang-flower pot (PF-1) and Penang-ground (PG-1) with the value of 1816±15.524 mg/ml, 2011±8.718 mg/ml and 2744±6.557 mg/ml respectively (Table 1).

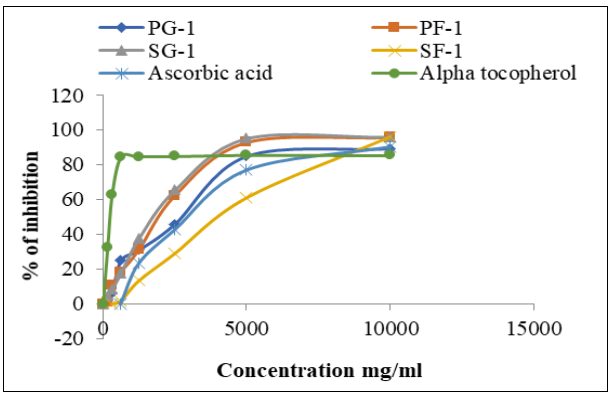

Fig.1: The percentage of inhibition of DPPH scavenging assay for hexane extract

The percentage of inhibition of all hexane extracts were increased as the concentration of the plant extract increased (Fig.1).

In chloroform extract, the highest percentage inhibition of DPPH was obtained in Penang-flower pot (PF-2) sample with the value of 1524±11.358mg/ml followed by the samples from Selangor-flower pot (SF-2), Selangor-ground (SG-2) and Penang-ground (PG-2) with the value of 1589±7.000 mg/ml, 1798±8.888 mg/ml and 2875±12.288 mg/ml respectively (Table 1). The percentage of inhibition of all chloroform extracts were increased as the concentration of the plant extract increased (Fig.2).

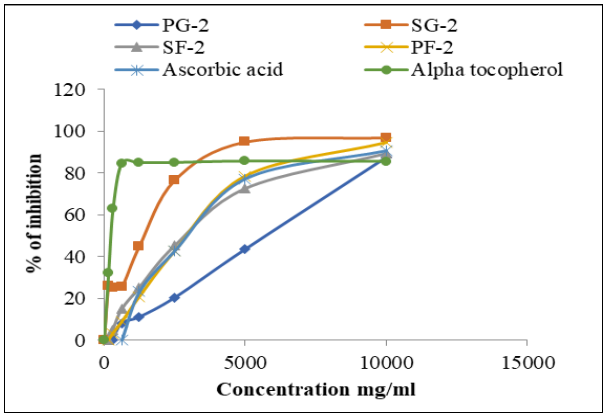

Fig.2: The percentage of inhibition of DPPH scavenging assay for chloroform extract

Meanwhile ethyl acetate of the whole plant extract presented the highest percentage of inhibition of 50% DPPH among the Selangor-flower pot (SF-3) sample, which exhibited the value of 1255±7.000mg/ml followed by the samples from Selangor-ground (SG-3), Penang-ground (PG-3) and Penang-flower pot (PF-3) with the value of 1279±7.810mg/ml, 1380±3.606 mg/ml and 1391±7.211mg/ml respectively (Table1). The percentage of inhibition of all ethyl acetate extracts were increased as the concentration of the plant extract increased (Fig.3).

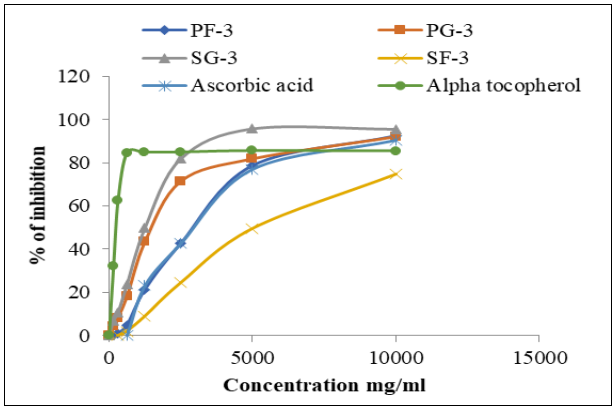

Fig.3: The percentage of inhibition of DPPH scavenging assay for ethyl acetate extract

As expected, the overall result for the antioxidant capacity study of DPPH assay showed the most potent antioxidant activity is in the methanol extract (Table 1). The methanol extract was found to inhibit 50% of DPPH at the concentration of 1108±5.568mg/ml in Selangor-ground sample (SG-4), which is the best antioxidant activity compared to the other extracts. Other samples from methanol extract showed the scavenging activity of 1241±5.292mg/ml in Penang-ground (PG-4), 1245±5.000mg/ml in Selangor-flower pot (SF-4) and 1432±4.583mg/ml in Penang-flower pot (PF-4) sample. The percentage of inhibition of all methanol extracts were increased as the concentration of the plant extract increased (Fig.4).

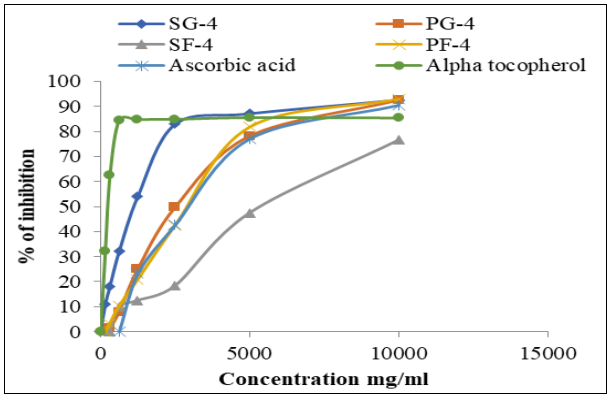

Fig.4: The percentage of inhibition of DPPH scavenging assay for methanol extract

However, from the above finding, the standards (ascorbic acid and alpha tocopherol) showed the best scavenging activity which at the value of 20.21±0.441 mg/ml and 14.71±0.062mg/ml, respectively as compared to tested extracts. It is important to calculate the value of IC_{50} in order to evaluate the potential of the plant extracts as antioxidant[41].

The second antioxidant assays focussed on *β*-carotene bleaching assay which is based on the bleaching of *β*-carotene orange colour due to the reaction with radicals formed by linoleic acid oxidation[42]. According to[43], *β*-carotene is used in *β*-carotene bleaching assay because it exhibits a strong biological activity. In this assay, alpha tocopherol and BHT have been used as standards which discovered the best percentage of inhibition (96.710%) in alpha tocopherol whilst BHT reported the percentage of inhibition of 81.990%. As shown in Table 1 and Fig.5, overall results for this assay presented that all sample extracts were moderately active between the percentages of inhibitions of 31.613% to 86.111% at 1mg/ml. The best radical scavengers was performed in ethyl acetate extract and it was found in Penang-ground sample (PG-3) which offered the highest percentage of inhibition (86.111%). The presence of antioxidant in extracts is important to neutralize the linoleate-free radical and other free radicals formed in the system and delay the

extent of *β*-carotene bleaching[44]. Therefore, the lowest *β*-carotene degradation rate indicated the highest antioxidant capacity[45, 46].

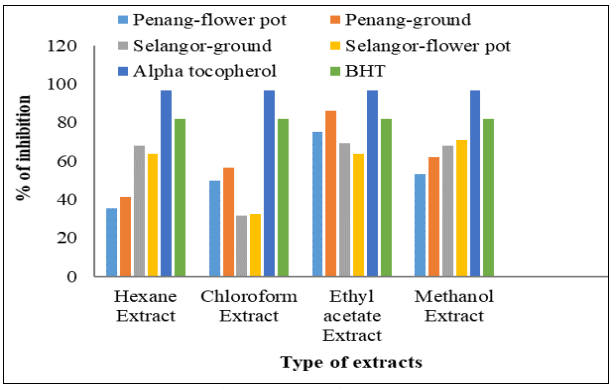

Fig.5: The percentage of inhibition of *β*-carotene bleaching assay

Contrary to the results from *β*-carotene bleaching assay, ORAC assay exhibited the highest scavenging activity in methanol extract (Table 1; Fig.6). In ORAC assay, antioxidant activity will be determined by the slowing of the fluorescence loss in the presence of antioxidant[47]. The overall result of ORAC assay showed that Penang-ground methanol extract (PG-4) offered the highest scavenging activity of 397.691µmol of Trolox equivalent/µl compared to others at the concentration of 1mg/ml. Meanwhile, the second highest antioxidant activity was evaluated in ethyl acetate extract from Penang-ground sample (PG-3) which exhibited the scavenging activity of 368.470 µmol of Trolox equivalent/µl. In addition, chloroform and hexane extract showed the scavenging activity value between 62.384 µmol to 24.263µmol of Trolox equivalent/µl.

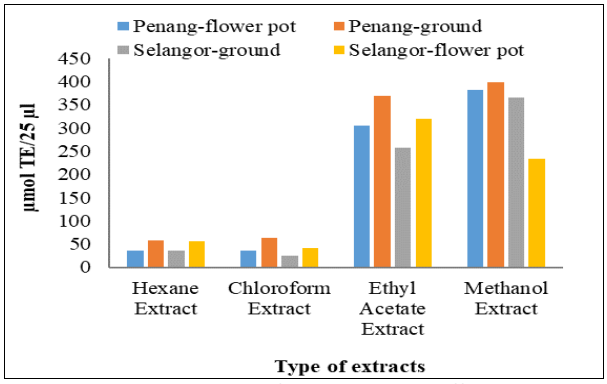

Fig.6: The Trolox equivalent of ORAC assay in different solvents extract

From the data, all antioxidant assays which are DPPH assay, *β*-carotene bleaching assay and ORAC assay showed different results in antioxidant capacity of *P. pellucida* for the whole plant extract. According to[48], antioxidant act in different mechanism of actions such as donation of hydrogen to free radicals, reducing power, metal chelating ability, free radical scavenging activity, inhibition of *β*-carotene bleaching and quenching singlet oxygen. Furthermore, the large differences in procedure, nature of the analytical samples and the principle of actions in DPPH and ORAC assay are the reasons why the studies cannot be compared to each other[49]. Thus several antioxidant assays are required to determine the antioxidant capacity[48].

In this study, the plant samples collected from the ground and flower pot have different kinds of growing condition. According to[50], the plant can grow healthy when it is well matched with its growing condition. Furthermore, the plant growth is depending on the abiotic factors (physical environmental conditions) such as pH of soil, pH of water, nutrient content, dissolved oxygen, re-oxidation potential[51], rainfall, climate and altitude[31] and also biotic factors such as human, animals, microorganisms and plants[52]. The availability of fertilizers in flower pot can alter the pH of the surrounding soil[33]. According to[32], soil nutrients and fertilizers can affect the plant yield and the quality of the plants. Meanwhile, the sample from the ground that surrounded by different kinds of plant was exposed to the soil with high organic matter in which can lead to alter the soil pH[53,33]. Soil pH plays an important role for the availability of nutrient in plant[34,35].

However, the analysed results between two locations; Penang and Selangor showed no significant difference as compared to the samples from Selangor except for DPPH scavenging assay which depicted a highly significant difference ($p<0.05$) in Penang (Table 2). Meanwhile, both sources of sample; ground and flower pot found to be no significant difference in all antioxidant assays (Table 3). Thus, the present findings found that both locations and sources do not affect the antioxidant capacities of this plant. This probably due to healthy condition of plant samples collected in both locations and sources.

Table 2: *In vitro* antioxidant assay analysis between two different locations

Assays	Locations	Mean ± SD
DPPH scavenging assay	Penang	1824.750±620.780*
	Selangor	1477.750±273.732
***B*-carotene bleaching assay**	Penang	57.323±16.540
	Selangor	58.503±16.139
ORAC assay	Penang	205.468±170.981
	Selangor	166.518±141.859

Note, * = the mean difference is significant at $p<0.05$
Values are expressed as mean±SD

Table 3: *In vitro* antioxidant assay analysis between two different sources

Assays	Sources	Mean ± SD
DPPH scavenging assay	Ground	1780.125±654.431
	Flower pot	1522.375±245.311
***B*-carotene bleaching assay**	Ground	60.279±16.845
	Flower pot	55.547±15.462
ORAC assay	Ground	196.091±167.002
	Flower pot	175.894±148.669

Note, * = the mean difference is significant at $p<0.05$
Values are expressed as mean±SD

Surprisingly, there was a significant difference ($p<0.05$) between hexane, chloroform, ethyl acetate and methanol extract of DPPH scavenging, *β*-carotene bleaching and ORAC assay (Table 4). The present study discovered that ethyl acetate and methanol exhibited the most potent antioxidant against DPPH, *β*-carotene bleaching and ORAC assays in the whole plant extract of *P. pellucida*. Previous study claimed that ethyl acetate is highly recommended as solvent for extraction that has capability to increase the effectiveness of antioxidant capacity from *P. pellucida*.[54]. Meanwhile, methanol is the most preferred polar solvent used in plant extraction[55], also has been found to be excellent in extracting antimicrobial activity related compound[25] and promotes the highest antioxidant activity in *P. pellucida*.[16, 56]. According to[57], methanol also has been discovered by many scientists in having a good record and effective as antioxidants.

Table 4: *In vitro* antioxidant assay analysis between four different solvents extraction

	DPPH scavenging assay	***B*-carotene bleaching assay**	**ORAC assay**
Hexane	2075.750±416.716*	52.030±15.392*	46.043±12.732*
Chloroform	1946.500±569.868*	42.571±11.994*	41.038±15.961*
Ethyl Acetate	1326.250±62.901*	73.585±9.070*	312.225±45.882*
Methanol	1256.500±120.561*	63.466±7.643*	344.665±75.660*

Note, ***** = the mean difference is significant at $p<0.05$
Values are expressed as mean±SD

CONCLUSION:

As conclusion, plant extraction is one of the most important parts to obtain good result at the end of the experiment. The selection of suitable solvents and methods for plant extraction which affected the plant's chemical compound is needed in order to explore the biological activities revealed by plant. However, the present study of *in vitro* antioxidant capacity in *P. pellucida* depicted that different locations and sources does not affect too much on the antioxidant activities of plant. In addition, there is a need to run more than one *in vitro* antioxidant assay to get more accurate results for the antioxidant activity in plant extract. Furthermore, each antioxidant assay has its own advantages and disadvantages due to the differences in the mechanism of action[58]. Further investigation on *P. pellucida* plant extract using other *in vitro* antioxidant assays is suggested to serve more information on the antioxidant activity of this plant for therapeutic purposes.

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