

ANTIOXIDANT ACTIVITIES OF *IN VITRO* SEEDLINGS OF *LYCIUM BARBARUM* (GOJI) BY DIPHENYL PICRYLHYDRAZYL (DPPH) ASSAY

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ABSTRACT

The antioxidant activities of *Lycium barbarum* (goji) methanolic extracts were analysed by DPPH assay. The extracts were prepared from different ages and plant parts of *in vitro* seedlings of goji. The plant parts involved were leaf, stem and root which were taken from four different *in vitro* developmental ages which were ranging from two to five months old *in vitro* seedlings. From the study, it was found that leaf and stem extracts of two months old seedlings exhibited the highest antioxidant activity with the EC₅₀ value of 0.08 mg/ml, comparable to that of ascorbic acid. However, the leaf of two months old extract showed the most outstanding activities by giving the highest percentage of inhibition (82.7%). On the other hand, leaf of five months old *in vitro* seedlings had been identified to be the least optimum extract whereby the EC₅₀ value was recorded to be 0.42 mg/ml. Generally, the effect of developmental ages on antioxidant properties was seen to be significant. Indeed, the results revealed that *L. barbarum* has an excellent activity of antioxidant and its usefulness to be consumed as a type of essential phytomedicines is proven.

Keywords: *Lycium barbarum*, Antioxidant activity, DPPH assay.

INTRODUCTION

Since ancient times plants have been acknowledged to be the source of medicines for human healthcare. Antioxidant property is one of the most valuable phytochemical values in plant to be used as natural remedies. Advanced knowledge related to dietary antioxidant benefits as disease prevention measures has initiated the efforts of discovering more antioxidant of natural sources to be utilized. Cells in humans and other organisms are constantly exposed to a variety of oxidizing agents which leads to the development of many diseases¹ including heart disease, cancer, and even aging^{2,3}. Many diseases are attributed to the oxidative damages caused by free radicals. Through various scientific findings, it has been proven that the consumption of antioxidants is useful in the prevention and treatment of a number of disorders related to oxidative damages⁴. In food industry, the synthetic antioxidants have been frequently used to be incorporated in the food products as a measure to control lipid oxidation reaction. For instance, butylated hydroxyl anisole (BHA), butylated hydroxyl toluene (BHT), propyl gallate and tertiary butyl hydroquinone are among the most widely used synthetic antioxidants. Nevertheless, the usage of those synthetic products has raised some questions pertaining to their possible risks of toxicity⁵. Therefore, this has initiated the urge for the searching and discovery of antioxidant substances from natural sources likewise in medicinal plants.

With regard to antioxidant properties, *L. barbarum* is one of the herbs that have been recognized to be the source of this phytomedicine. It belongs to the family of Solanaceae and found wild around the hills in the Ningxia region of China, and in remote areas of central China near inner Mongolia⁶. Commonly known as goji, *L. barbarum* has long been consumed in Chinese cuisines and traditional medicines. Goji has a long history of medicinal use especially among Chinese tribes^{7,8,9}. It has been recognized to be an exotic super fruit and very often included in foods especially in preparing Chinese tonic soups. In Malaysia, only the imported dried goji berries are consumed since this species is not available locally.

L. barbarum polysaccharide (LBP) is reported to have efficient immunomodulatory properties and inhibiting tumour growth^{2,9}. Another interesting finding reported by Li *et al.*¹⁰ had proven that the polysaccharide of *L. barbarum* is effective in counter-acting oxidative stress damage. In addition to this, Feng *et al.*⁷ indicated that *L. barbarum* is also effective in providing protection for retina from oxidant injury in diabetic subjects.

However, the capacity of antioxidant activity in each plant part and age may vary and need to be assessed. The potential of natural

sources from plant materials to be harnessed and formulated in pharmaceutical preparations has initiated the attempt to discover and analyse which plant parts of a certain species are bearing the outstanding amount of antioxidative activities. Same quest of knowledge related to this has also initiated the effort to identify the optimum plant age with paramount contents of antioxidants. Therefore, this study was aimed at achieving those goals. However, as goji is not locally found in Malaysia, thus the acquisition of extracts can only be made through *in vitro* seed germination whereby the seeds were obtained from the imported dried goji berries. Thus, the *in vitro* germination of the species was carried out accordingly beforehand to produce *in vitro* seedlings to serve as the source of plant materials.

In this study, the method of DPPH assay was used and the comparative antioxidant assessments were conducted mainly to compare the antioxidant capacity of this species with respect to its different ages and plant parts. In such assay, DPPH is best corresponds to a model radical which will be reduced by antioxidant properties derived from the extracts. DPPH is a relatively stable free radical and can be reduced by electron-rich radical scavengers from medicinal plant extracts.

MATERIALS AND METHOD

Chemicals

DPPH free radical reagent (Sigma), methanol for analysis Emsure® (Merck), and L-Ascorbic acid (R&M).

Collection of plant materials

The plant materials were taken according to the growth time interval of the *in vitro* seedlings of *L. barbarum*. They separated into their different plant parts namely leaf, stem and root for each developmental age of two, three, four and five months old *in vitro* seedlings.

Extraction of plant samples

The method of extraction was conducted by referring to Azizah *et al.*¹¹ with some modifications. Some of the samples (the root parts) were cleaned under running tap water to remove the remaining germination media. Then they were dried at 50°C for 48 hours in the oven. Afterwards, the plant samples were weighed and crushed into coarse powder. Extraction was carried out by soaking 100 mg of plant sample in 50 ml of 70% aqueous methanol for 120 minutes by using orbital shaker and filtered using Whatman filter paper. The resulting filtrates obtained were used for the assay.

Determination of antioxidant activities by diphenyl picrylhydrazyl (DPPH) assay

This assay was conducted according to Nurliyana *et al.*,¹² with some modifications. An amount of 1.0 ml extract was added to 2.0 ml of 0.15 mM DPPH. They were allowed to stand for 20 minutes before the absorbance readings were taken at 517 nm by using UV/Vis spectrophotometer. Ascorbic acid was used as positive standard. The tests were run in duplicate and the readings were averaged. Percentage of Inhibition (% In) of DPPH radical by test compounds was determined by the following formula.

$$\% \text{ Inhibition} = \frac{\text{Absorbance}_{(\text{control})} - \text{Absorbance}_{(\text{extract})}}{\text{Absorbance}_{(\text{control})}} \times 100\%$$

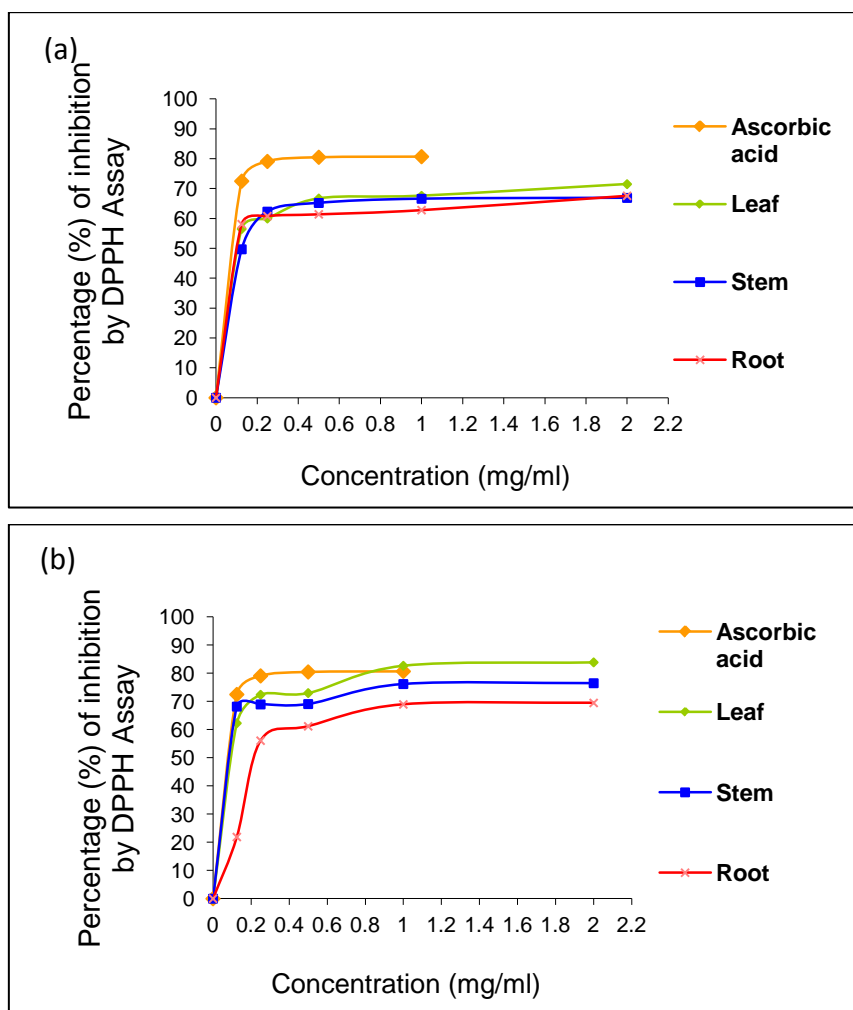
Analysis of results

The Percentage of Inhibitions (% In) values were used to produce graphical plots of dose-response curve. The graphs plotted were constructed between the scavenging activities (% In) versus the extracts' concentrations. The EC_{50} values were determined from the curves constructed for each month by graphical interpolation of the concentration of extract at which the % In is 50%. The activity is expressed as effective concentration EC_{50} whereby it signifies the effective concentration of extract required to scavenge DPPH radical by 50%¹³. The lower the EC_{50} value, the greater the free radical scavenging activity that the extract possesses. The SPSS 17.0 software was used for statistical analysis and the results were analysed by ANOVA. The level of $p < 0.05$ was considered to be significant.

RESULTS AND DISCUSSION

In this study, the antioxidative properties of *L. barbarum* were measured spectrophotometrically by DPPH assay. DPPH (diphenyl picrylhydrazyl) assay is the most widely reported method for screening of antioxidant activity of many plant drugs¹⁴. DPPH assay method is preferred due to its simplicity, convenience and time-saving properties. The concept involved in this method is particularly focusing on the ability of the tested extract to scavenge a stable DPPH free radical. The DPPH assay method is preferred due to its convenience which can evaluate the activities in a relatively short duration of time.

The basis of DPPH assay activity relies on the concept of delocalisation of spare electron over the molecule of DPPH. Upon addition of substance with hydrogen donor property, DPPH will undergo reduction process and the colour of solution changes from deep violet to yellow¹⁵. The extract which functions as antioxidant reacted with DPPH, chemically named after 1-diphenyl-2-picrylhydrazyl thus resulted in the formation of 1-diphenyl-2-picrylhydrazine¹⁶. This conversion is visible by the change of colour from deep violet to pale yellow or almost colourless. The disappearance of DPPH radical chromogens reflects the presence of antioxidant in the tested extracts¹⁷. The colour changes after reduction can be quantified by its decrease of absorbance at wavelength 517 nm. The quantified reduction of absorbance reflected the reduction capability of DPPH radical by antioxidative agents, namely the tested extracts. In this study, ascorbic acid (Vitamin C) was used as positive standard. It is a well known biological antioxidant and it exerts its antioxidative properties as a potent free radicals scavenger by acting as a chain-breaking scavenger for peroxy radicals^{18,19}.



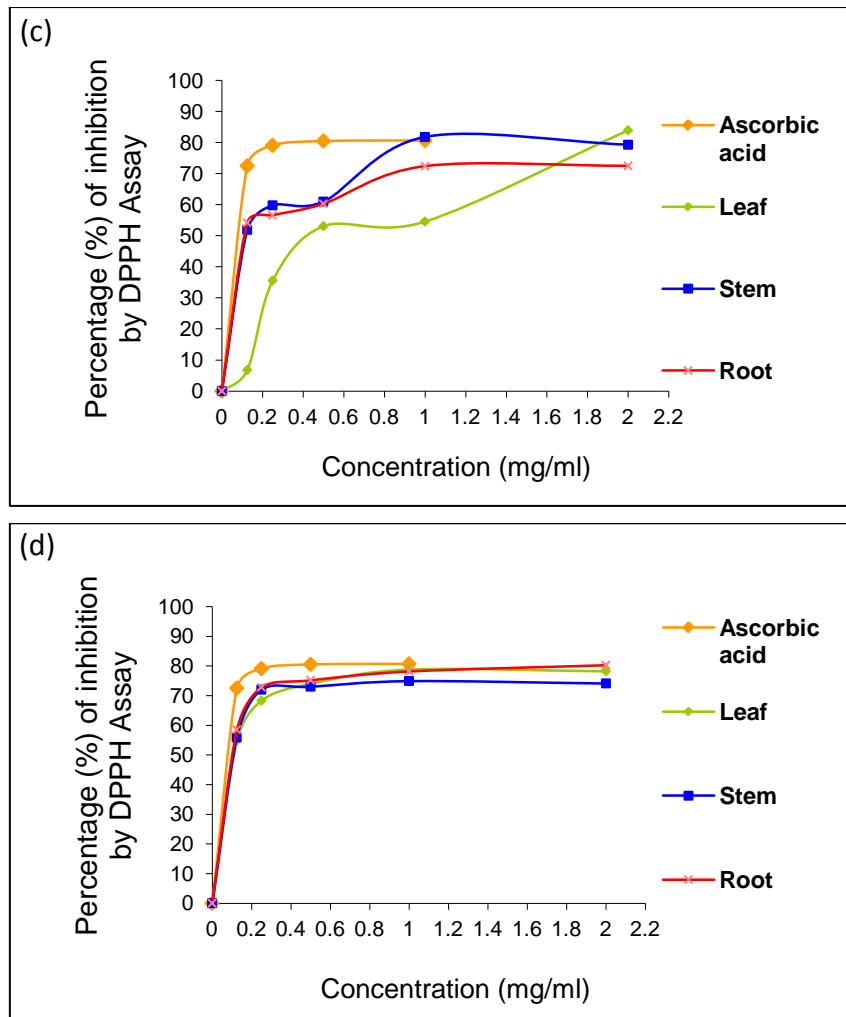


Fig. 1: Scavenging activity of methanolic extracts of (a) two months old, (b) three months old, (c) four months old, and (d) five months old *in vitro* seedlings of *L. barbarum* on DPPH radical.

Generally, from the results obtained, all extracts were seen to have a considerably good antioxidant activity. A graph was constructed for each developmental age involved in the study to figure out the EC_{50} values from graphical interpolation [Figure 1 (a) to (d)]. The EC_{50} serves as a parameter for the interpretation of the results in DPPH assay. It reflects the meaning of efficient concentration of the substrate which is required to cause an amount of 50% loss in DPPH radical activity¹⁵. From the dose-response curve constructed, it was found that the scavenging activities of all extracts were depending on the concentration of the extracts. The activities were seen to increase with the increase of extracts' concentrations.

The bar charts in Figure 2 presented according to the type of plant parts involved. Particularly in leaf extracts, the highest EC_{50} value was found in the five months old leaf extract (0.42 mg/ml) thus suggesting that the leaf extract at that particular age would exhibit the lowest ability of free radical scavenging activity as compared to its younger counterparts. In contrast with the two months old extracts, the EC_{50} value was found to be the lowest (0.08 mg/ml) hence signifying its radical scavenging activity as the greatest among other extracts assessed. Meanwhile the EC_{50} value for three and four

months old leaf extracts were 0.10 mg/ml and 0.11 mg/ml respectively.

As for the stem extracts, the EC_{50} values were seen to be almost uniformly scored. Each developmental age was seen to have a high antioxidant activity. The two months old stem extract exhibited the lowest EC_{50} value with 0.08 mg/ml comparable to that of ascorbic acid as positive standard. Meanwhile, three and five months old stem extracts scored greatest EC_{50} value (0.12 mg/ml) while the four months old stem extract exhibited 0.11 mg/ml EC_{50} value as intermediate score for stem extracts.

In contrast with leaf and stems whereby the two months old extracts had given the greatest free radical scavenging activity results, the condition was the other way around in root wherein the two months old extracts exhibited the lowest activity as free radical scavenger with the EC_{50} value of 0.22 mg/ml. On the other hand, three and four months old root extracts exhibited highest activity with the EC_{50} value of 0.10 mg/ml while the five months old extract had demonstrated a bit lower activity than those of three and four months old *in vitro* seedlings, thus ranked itself as an intermediate scorer with 0.11 mg/ml EC_{50} reading.

Table 1: The EC_{50} values (mg/ml) of each plant part in each developmental age

| Developmental ages (months old) | EC_{50} values (mg/ml) according to plant parts | | |
|---------------------------------|---|------|------|
| | Leaf | Stem | Root |
| 2 | 0.08 | 0.08 | 0.22 |
| 3 | 0.10 | 0.12 | 0.10 |
| 4 | 0.11 | 0.11 | 0.10 |
| 5 | 0.42 | 0.12 | 0.11 |

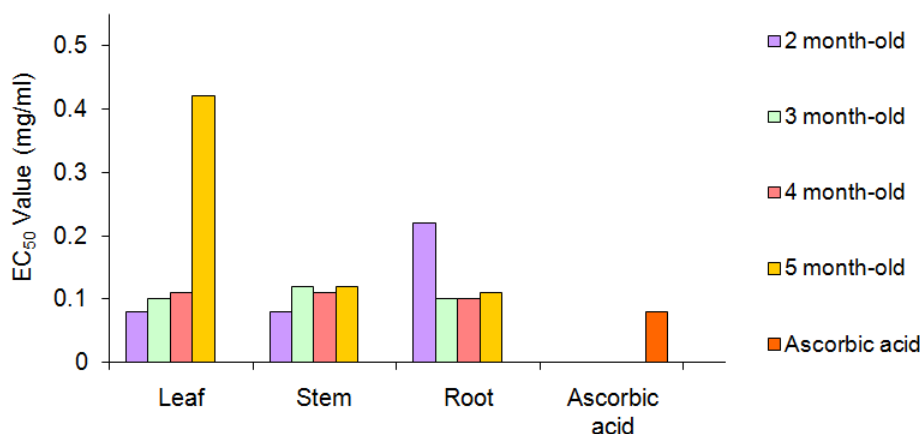


Fig. 2: The EC₅₀ value according to plant parts

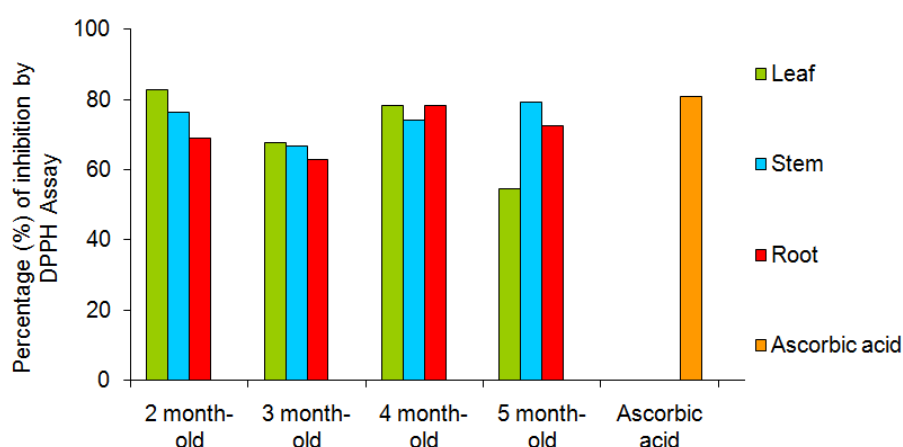


Fig. 3: The scavenging activity expressed as percentage of inhibition (% In) of methanolic extracts of two to five months old *in vitro* seedlings of *L. barbarum* on DPPH radical at the concentration of 1000 ppm (1.0 mg/ml)

After assessing the antioxidant properties of *L. barbarum* by DPPH assay, it was found that almost all parts of *L. barbarum* plant possessing considerable activity of antioxidant with the two months old leaf and stem exerted scavenging activity as good as ascorbic acid in terms of their EC₅₀ values. Nevertheless, among all the extracts assessed, the most optimum extract which possesses optimum antioxidant activity was seen to be in the two months old leaf *L. barbarum* which exhibited the lowest EC₅₀ values comparable to that of ascorbic acid. In addition to that, the percentage of inhibition exhibited by this extract at 1000 ppm (Figure 3) had proven its excellent antioxidant potency by showing 82.7% percentage of inhibition, which was found to be a bit higher than that of ascorbic acid (80.5%) as a positive standard.

Developmental age was seen to have a significant effect on antioxidant properties. From the results of the study particularly in roots, the lowest antioxidant activity was recorded in the youngest extract of two months old plantlets. In contrast with the roots, the pattern of free radical scavenging activities in leaf was seen in a decreasing trend over times and it was found that the younger the leaf, the greater the antioxidant properties by referring to the EC₅₀ values of the extracts. As shown in this study, the leaves of the two months old *L. barbarum* were the most optimum extract with the greatest antioxidant activities.

According to the statistical analysis done on the EC₅₀ value by ANOVA, which were considering the effects of samples' ages and plant parts on the activity of antioxidant of the species, a significant difference ($p < 0.05$) had been highlighted in the factor of age. Thus,

the findings verified that the factor of age from which the extracts obtained significantly influenced the antioxidant activity. On the other hand, the results from statistical analysis had shown the insignificant differences in the factor of varying plant parts towards the activity of antioxidant ($p > 0.05$).

A finding by Wang and Lin²⁰ had also proven that leaves possess an outstanding level of antioxidants. Their study for antioxidant activity was conducted by using ORAC (Oxygen Radical Absorbance Capacity) assay and they found that the ORAC values were high in all leaf extracts of blackberry (*Rubus* sp.), red raspberry (*Rubus idaeus* L.), black raspberry (*Rubus occidentalis* L.) and strawberry (*Fragaria x ananassa* D.). In addition, it had been noted that as the leaves become older, the ORAC values and the total phenolic contents decreased. Therefore, this had resulted in the decrease in antioxidant activity as well, since there was a linear correlation between total phenolic content and ORAC activity.

Therefore, the results from the present study clearly suggested that *L. barbarum* may serve as an excellent source of antioxidants and bearing health promoting factors not only by the consumption of the berries, but also by optimally consuming almost all parts of the plants.

Indeed, from this study, it can be deduced that almost all parts of *L. barbarum* plant possessing considerable antioxidant activity with the two months old leaf and stem exerted scavenging activity as good as ascorbic acid in terms of their EC₅₀ values. These findings are also beneficial in providing a useful benchmark in determining the optimum age and plant part from this species at its best, most

promising antioxidative effects. In addition, it may serve as a basis for even more extensive researches to be done on this species with the focus of interest directed towards its phytomedicinal values, hence would be incorporated into health-promoting supplementary foods and pharmaceutical preparations.

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